DETECTION OF PROSTATE SPECIFIC mRNAs IN CIRCULATION AND IN PROSTATIC TISSUE

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Academic Dissertation

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TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I. Jaakkola S, Vornanen T, Leinonen J, Rannikko S and Stenman UH. Detection of prostatic cells in peripheral blood: correlation with serum concentrations of prostate-specific antigen. Clin Chem. 1995; 4: 182-6.

II. Lintula S and Stenman UH. The expression of prostate-specific membrane antigen in peripheral blood leukocytes. J Urol. 1997; 157: 1969-72.

III. Lintula S, Vesalainen S, Rannikko A, Zhang WM, Finne P, Stenman J and Stenman UH. Quantification of prostate specific antigen mRNA levels in circulation after prostatic surgery and endocrine treatment by quantitative reverse transcription-polymerase chain reaction. Scand J Clin Lab Invest. 2004; 64: 93-100.

IV. Lintula S, Stenman J, Bjartell A, Nordlig S and Stenman UH. Relative concentrations of hK2/PSA mRNA in benign and malignant prostatic tissue. Prostate 2005; 63: 324-329.

ABBREVIATIONS

A2M	alpha-2-macroglobulin
ACT	alpha-2-antichymotrypsin
AP	alkaline phosphatase
API	alpha-1-protease inhibitor
AMARC	alpha-methylacyl-coenzyme A racemase gene
AR	androgen receptor
ARE	androgen responsive element
BPH	benign prostatic hyperplasia
cDNA	complementary deoxyribonucleic acid
CTC	circulating tumor cells
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EST	expressed sequence tag
HER-2/neu	avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2)
hK2	human glandular kallikrein 2
IGF-1	insulin-like growth factor 1
IS	internal standard
kb	kilobase
kD	kilodalton
mRNA	messenger ribonucleic acid
PCa	prostate carcinoma
PCR	polymerase chain reaction
PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen
PSM/PSMA	prostate specific membrane antigen
PTEN	phosphatase and tensin homolog gene
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RB1	retinoblastoma 1 gene
RNA	ribonucleic acid
RP	radical prostatectomy
RT-PCR	reverse transcription-polymerase chain reaction
TNM	tumor-node-metastasis
TRUS	transrectal ultrasound
TURP	transurethral resection of the prostate

ABSTRACT

Occurrence of mRNA specific for a certain tissue in peripheral blood is thought to indicate the presence of circulating cancer cells and metastatic spread of a tumor originating from this tissue. The aim of this work was to study the mRNA expression of prostate specific genes in prostatic tissue as well as in blood from prostate cancer (PCa) patients. As prostate specific genes we studied prostate specific antigen (PSA) and prostate specific membrane antigen (PSM). We also investigated the relative levels of human glandular kallikrein (hK2) and PSA mRNAs in malignant and benign prostatic tissue.

Expression of PSA mRNA in peripheral blood was found by reverse transcriptase-polymerase chain reaction (RT-PCR) in 9 out of 18 PCa patients with metastatic disease but in none of 7 patients without metastases. Negative results in patients with metastatic disease were associated with successful endocrine therapy and low levels of serum PSA, and the correlation between serum concentrations of PSA and the presence of PSA mRNA in peripheral blood was statistically significant. PSA mRNA was not found in four patients with benign prostatic hyperplasia (BPH), other types of cancer or in six healthy male and three female controls. Thus the occurrence of PSA mRNA in peripheral blood was associated with metastatic PCa.

PSM mRNA was detected in blood from 18 out of 24 PCa patients and 12 out of 13 healthy donors and in the leukocyte fraction of normal blood cells. PSM expression could not be detected in erythroblasts, platelets, K-562, U-937, HL-60 or Jurkat cell lines. Our results indicate that sensitive nested RT-PCR method detects PSM mRNA in the leukocyte fraction of normal blood, which is probably caused by a leaky promoter for the PSM gene. Development of quantitative RT-PCR assay to differentiate PSM mRNA expression derived from circulating cancer cells from background expression in blood cells seems to be necessary in order to make PSM useful for detection of PCa.

By using a quantitative RT-PCR (qRT-PCR) method the influence of prostatic surgery and endocrine treatment on prostatic cells in circulation was studied in serial samples. A competitive internal mRNA standard was used for quantification of absolute concentrations of PSA mRNA. The detection limit of the assay was 7 copies of PSA mRNA. Based on blood levels measured from 88 control subjects the cutoff level for circulating PSA mRNA was defined as 26 copies per mL blood. A total of 56 patients scheduled for biopsy, radical prostatectomy (RP), transurethral resection of the prostate (TURP), orchiectomy or total androgen blockade were included in the study. PSA mRNA levels in circulation were measured before, during and up to 26 weeks after these procedures by a quantitative RT-PCR method. Immediately after RP or TURP, the PSA mRNA levels increased in 27% and 29% of the samples, respectively. Orchiectomy also caused a moderate increase in 25% of the samples. After prostate biopsy, two samples out of 15 became positive. Detectable PSA mRNA levels became undetectable after 3 days to 6 months. No significant correlation was found between PCR positivity and clinical characteristics of the patients. The PSA mRNA level varied from 26 to 956 copies/mL blood. At sensitivity levels close to the detection limit the accuracy of amplification based quantification and detection systems is impaired by stochastic factors arising during sampling. This phenomenon is likely to explain the variable results published in earlier reports.

Expression of hK2 and PSA is regulated by similar mechanisms but changes in their relative expression have been observed in prostate cancer. We therefore determined the relative levels of PSA and hK2 mRNA in benign and malignant prostate tissue using a

quantitative method, in which mRNAs of PSA and hK2 are reverse transcribed and amplified in one reaction with the same primers. The variation in the ratio of hK2/PSA mRNA was remarkably small, the difference between the highest and lowest values being 3-fold, but the ratio of hK2/PSA mRNA was significantly higher in cancerous than in benign prostatic tissue. This new quantitative RT-PCR technique facilitates very accurate quantification of the relative mRNA levels of similar genes.

1. REVIEW OF THE LITERATURE

1.1. PROSTATE CANCER

Prostate cancer is the most common cancer in men in most industrialized countries including Finland (Finnish Cancer Registry, 2004) and the second most common cause of cancer-related death in men in the United States (Landis et al., 1999). In Asian countries, the incidence and mortality of prostate cancer is only 4% of those observed in the U.S. (Whittemore, 1989). Factors other than genetic, e.g. diet, have been suggested to be important in promoting or preventing the development of prostate cancer (Parkin and Muir, 1992).

Although 30-50% of men above the age of 50 have microscopic prostate cancer (Franks, 1956), only 9-11% develop clinical disease and under 5% die of the disease (Seidman et al., 1985). Clinically insignificant cancers are typically found in patients in their eight or ninth decade of life. Distinct from this group are cancers with aggressive growth pattern and metastatic potential that develop in younger patients (Albertsen et al., 1998). A family history of prostate cancer is found only in 5-10% of all prostate cancers (Carter et al., 1992).

Over 98% of the prostate cancers are epithelial adenocarcinomas (Dube et al., 1973). The extent of prostate adeno-carcinoma is described by the TNM (tumor, node, metastasis) staging system (Table 1), which takes into account the size of the tumor within the prostate (T), the presence of lymph node spread (N), and documented evidence of the metastatic spread to other organs (M) (Schroder et al., 1992; Vaughan et al., 1998). Prostate cancer is graded on the basis of the glandular differentiation pattern of the tumor. The most widely used system is the Gleason grading system (Gleason, 1992) (Table 2). The most common pattern of dedifferentiation is assigned the primary grade and the second most common pattern defines the secondary grade. The Gleason score or Gleason sum is obtained by addition of the primary and secondary grade (Gleason and Mellinger, 1974). Another grading system, the WHO system, classifies the tumor into three categories: well (G1), moderately (G2) or poorly (G3) differentiated (Mostofi, 1975).

Prostate cancer mostly gives rise to clinical symptoms only after it has spread through the prostatic capsule or has metastasized to lymph nodes or bones. Curative treatment of prostate cancer is limited to the time, when the tumor still is organ-confined. In most patients the disease remains organ-confined for years (Schmid et al., 1993). Especially for the patients with rapidly progressing disease, early diagnosis is important. Determination of total serum PSA followed by biopsy is the most common method for early diagnosis of prostate cancer. Digital rectal examination is also used but it has a low predictive value and many clinically significant cancers may be missed (Schroder et al., 2000). By trasrectal ultrasound (TRUS) it is possible to get a more precise estimate of prostate volume. TRUS is used to determine the size, shape and internal echo image of the prostate gland (Babaian et al., 1992a; Terris and Stamey 1991; Watanabe et al., 1975). A limitation of TRUS is the inability to reveal pelvic lymph nodes, which can be observed by computerized tomography (CT) or magnetic resonance imaging (MRI). Prostate cancer preferentially metastasizes to bone. Radionuclide bone scan is the primary method to detect bone metastases. If serum PSA is below 10 μg/L, the risk for bone metastases is <1% (Gleave et al., 1996). A bone scan is recommended to patients with bone pain, a PSA concentration over 10 μg/L, or increased concentration of serum alkaline phosphatase (AP) (Suomen Urologiyhdis-tys ry, 1999).

Table 1. The TNM system for staging of prostate cancer

Primary tumor (T)		
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1		Clinically unapparent tumor
	T1a	Tumor found incidentally at TURP: 5% or less of tissue is cancerous
	T1b	Tumor found incidentally at TURP: 5% or more of tissue is cancerous
	T1c	Tumor found in prostate biopsy because of elevated serum PSA
T2		Palpable tumor confiend within the prostate
	T2a	Tumor involves half of lobe or less
	T2b	Tumor involves more than half of one lobe
	T2c	Tumor involves both lobes
T3		Tumor extends through the prostatic capsule
	T3a	Unilateral extracapsular extension
	T3b	Bilateral extracapsular extension
	T3c	Tumor involves seminal vesicles
T4		Tumor is fixed or invades adjacent structures other than seminal vesicles
	T4a	Tumor invades bladder neck and/or external sphincter and/or rectum
	T4b	Tumor invades levator muscles and/or is fixed to pelvic wall
Regional lymph nodes (N)		
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastases
N1-3		Regional lymph nodes
Distant metastases (M)		
MX		Presence of distant metastases cannot be assessed
M0		No distant metastases
M1a-c		Distant metastases

References: Schröder et al. 1992, Vaughn et al. 1998

Table 2. The Gleason grading system of prostate cancer.

Grade	Description
1	Simple round glands, close-packed in rounded masses with well-defined edges
2	Simple rounded glands, loosely packed in vague, rounded masses with loosely defined edges
3A	Medium sized single glands of irregular shape and irregular spacing with ill-defined infiltrating edges
3B	Very similar to 3A, but small to very small glands, which must not form significant chains or cords
3C	papillary and cribiform epithelium in smooth, rounded cylinders and masses; no necrosis
4A	Small, medium, or large glands fused into cords, chains, or ragged, infiltrating masses
4B	Very similar to 4A, but with many large clear cells, sometimes resembling "hypernephroma"
5A	papillary and cribiform epithelium in smooth, rounded masses, more solid than 3C and with central necrosis
5B	Anaplastic adenocarcinoma in ragged sheets

Reference: Gleason 1992

For organ confined prostate tumor (T1-T2N0M0) the most effective therapy is radical prostatectomy or radiotherapy (Catalona et al., 1999; Shipley et al., 1999). Due to the risk of complications, radical prostatectomy is usually used only when the expected lifetime of the patient is at least ten years. Alternative therapy is radical radiotherapy, which may be curative also when the cancer has spread locally outside the prostatic capsule (T1-T3N0M0). If the tumor is well differentiated and organ confined, 10-year survival rate after radical prostatectomy or radiotherapy is 90-94% (Catalona, 1994; Lu-Yao and Yao, 1997). However, about 30% of patients initially diagnosed with clinically localized prostate cancer, have non-organ confined disease and suffer from recurrent cancer after radical prostatectomy (Partin et al., 1993).

The aim of radical prostatectomy is to remove the cancer tissue entirely, but if the disease is diagnosed when extraprostatic or metastatic, hormonal therapy is mainly used (Catalona, 1994). Well differentiated prostate cancer cells are dependent on androgens and endocrine therapy aims at androgen deprivation of the tumor. This can be achieved by orchiectomy or by administration of estrogens, antiandrogens or synthetic agonists of gonadotropin releasing hormone (GnRH). However, endocrine treatment usually leads to androgen-independent disease within 3-5 years. This transition is thought to occur through selection of androgen-independent cells already existing in the tumor prior to androgen deprivation (Isaacs and Coffey, 1981). The expected lifetime of patients with metastases is 2-3 years, but if the cancer is androgen-independent, it is less than one year (Tannock et al., 1996).

In the past, the serum prostatic acid phophatase (PAP) level was used as a marker to detect prostate cancer. Although somewhat useful in monitoring patients with advanced disease, it has low sensitivity in localized disease. In recent years, examination of PAP has been replaced by PSA (Seamonds et al., 1986; Stamey et al., 1987).

Circulating tumor cells (CTC) can be detected at extreamely low levels in the blood of patients with carcinomas. The number of CTC may represent tumor burden, and changes in CTC numbers could offer a way to evaluate the effectiveness of a given treatment. The occurrence of CTC has been studied in patients with various cancers, including PCa, by using immunochemical methods and recently more by using RT-PCR to detect organ-specific mRNA in blood.

1.2. PROSTATE SPECIFIC ANTIGEN (PSA) AND HUMAN GLANDULAR KALLIKREIN (hK2)

1.2.1. Genes, mRNAs and proteins

Prostate-specific antigen (PSA/hK3) and human glandular kallikrein 2 (hGK1/hK2) and the corresponding genes (*KLK3* and *KLK2*, respectively) belong to the human tissue kallikrein gene family (Table 3) (Lundwall and Lilja, 1987; Schedlich et al., 1987), which comprises 15 tandemly localized genes in chromosomal locus 19q13.4 (Diamandis and Yousef, 2002). Tissue kallikreins form a subgroup in the serine protease enzyme family. Of the 176 serine protease genes within the human genome, this family represents the largest continuous cluster (Puente et al., 2003). Recently the first human kallikrein pseudogene, *YKLK1* has been identified in the same locus (Yousef et al., 2004). The sequence similarity between kallikreins at the DNA and amino acid levels is 40-80% (Diamandis and Yousef, 2002). The similarity between the coding nucleotides of *KLK2* and *KLK3* is 86% (Schedlich et al., 1988), which is the highest in the kallikrein family (Yousef et al., 2000). In addition to the protein coding sequences, the non-coding sequences, including 5' ends and introns, are highly conserved in *KLK2* and *KLK3* (Schedlich et al., 1987;

Table 3. Human kallikrein gene family.

Gene	No. of transcript variants	Protein	Other names	Specificity
KLK1	3	hK1	hPRK	trypsin-like
KLK2	5	hK2	hGK-1	trypsin-like
KLK3	13	hK3	PSA, APS	chymotrypsin-like
KLK4	8	hK4	prostase, KLK-L1, EMSP1, PRSS17	trypsin-like
KLK5	5	hK5	KLK-L2	trypsin-like
KLK6	6	hK6	Zyme, Neurosin, Protease M	trypsin-like
KLK7	3	hK7	HSCCE, PRSSL1	chymotrypsin-like
KLK8	4	hK8	Neuropsin, Ovasin	trypsin-like
KLK9	2	hK9	KLK-L3	chymotrypsin-like
KLK10	1	hK10	NES1, PRSSL1	trypsin-like
KLK11	3	hK11	TLSP, Hippostasin, PRSS20	trypsin-like
KLK12	3	hK12	KLK-L5	trypsin-like
KLK13	8	hK13	KLK-L4	trypsin-like
KLK14	2	hK14	KLK-L6	trypsin-like
KLK15	5	hK15	Prostinogen, HSRNASPH	trypsin-like

Modified from Borgono et al. (2004)

Riegman et al., 1989; Lundwall, 1989). Both genes have 5 coding exons and 4 introns (Riegman et al., 1989; Liu et al., 1999). The PSA gene produces at least 13 and the hK2 gene 5 different mRNA transcripts. They result from alternative splicing or/and alternative polyadenylation of these genes (Lundwall and Lilja, 1987; Riegman et al., 1989; Monne et al., 1994; Tanaka et al., 2000; Heuze et al., 1999; Heuze-Vourc'h et al., 2003; Heuze-Vourc'h et al., 2000; David et al., 2002; Schedlich et al., 1987; Riegman et al., 1991; Liu et al., 1999). The wild type PSA mRNA is 1464 bp and hK2 mRNA is 901 bp long. They both code for glycoproteins with a 17 amino acid signal peptide and a 7 amino acid propeptide, which is removed upon activation of the proenzyme to the mature 237 amino acid protein (Lundwall and Lilja, 1987; Schedlich et al., 1987).

1.2.2. Expression and function

PSA was first isolated from seminal plasma and called gamma-seminoprotein (Hara et al., 1971). Later it was isolated from prostatic tissue and named PSA (Wang et al., 1979). PSA and hK2 are mainly expressed by the prostatic epithelium, and the level of hK2 mRNA has been found to be 10-50% of that of PSA mRNA in normal prostatic tissue (Chapdelaine et al., 1988; Henttu et al., 1990; Young et al., 1992). In seminal plasma the hK2 levels are only about 1% of the PSA levels (Deperthes et al., 1995). Low level expression of both genes has also been found in other tissues like endometrium, normal breast and breast cancer, pituitary tissue and lung cancer (Clements and Mukhtar, 1994; Diamandis et al., 1994; Yu et al., 1996; Hsieh et al., 1997; Clements et al., 1996; Levesque et al., 1995). In the Unigene database it is also possible to find expressed sequence tags (EST) encoding PSA in the sciatic nerve, hematopoietic stem cells (CD34+/38-), skeletal muscle and brain (nervous tumor) as well as adrenal gland and liver. ESTs for hK2 are also found in the sciatic nerve, skeletal muscle and hematopoietic stem cells (http://www.ncbi.nlm.nih.gov/UniGene/).
By in situ hybridization, immunohistochemistry and RT-PCR, PSA and hK2 were recently found to be expressed also in ileum, jejunum, epididymis, seminal vesicle and skin (Olsson et al., 2005). PSA and hK2 are up-regulated by androgens, especially by dihydrotestosterone and the synthetic androgen mibolerone (Young et al., 1991; Young et al., 1992; Wolf et al., 1992; Henttu et al., 1992). Androgens exert

their function via the androgen receptor, which interacts with specific DNA sequences (androgen responsive element, ARE) in the PSA and hK2 genes (Carson-Jurica et al., 1990). Mutations of the androgen receptor (AR) gene in the human prostatic adenocarcinoma cell line, LNCaP, have been shown to cause expression of PSA also by other steroids than androgens (Montgomery et al., 1992).

The physiological function of PSA is to digest the gel forming after ejaculation (Lilja, 1985). Another possible role of PSA is to cleave insulin-like growth factor binding protein 3 (IGFBP-3) causing release of insulin like growth factor 1 (IGF-1), which can stimulate the growth of prostate cancer (Cohen et al., 1991). PSA has also been shown to degrade extra-cellular matrix glycoproteins fibronectin and laminin, which could facilitate invasion by prostate cancer cells (Webber et al., 1995). It may also facilitate bone metastases by activating latent trans-forming growth factor-beta (Killian et al., 1993). PSA may on the other hand suppress tumor growth by converting plasminogen to biologically active angiostatin, a potent inhibitor of angiogenesis (Heidtmann et al., 1999). PSA has been shown to inhibit endothelial cell proliferation, migration, and invasion, which may explain, in part, the naturally slow progression of prostate cancer (Fortier et al., 1999). A role of PSA as a tumor suppressor also is supported by the finding that low expression of tissue PSA is related to adverse prognosis (Stege et al., 2000).

Active hK2 has the capacity to convert the proenzyme form of PSA (pro-PSA) to mature, enzymatically active PSA (Kumar et al., 1997; Lovgren et al., 1997). HK2 can also activate pro-hK2 suggesting that maturation of pro-hK2 to enzymatically active hK2 involves autoactivation (Denmeade et al., 2001). In addition, hK2 has been shown to convert latent prourokinase-type plasminogen activator to active urokinase-type plasminogen activator (uPA) and to inactivate its primary inhibitor PAI-1 (Frenette et al., 1997; Mikolajczyk et al., 1999). Furthermore, an increased expression of hK2 has been observed in poorly differentiated prostate cancer (Darson et al., 1999; Darson et al., 1997).

1.2.3. Clinical aspects

PSA is normally secreted into seminal fluid and only small amounts leak out into circulation. The PSA levels in patients without prostate cancer correlate with prostate volume (Babaian et al., 1992b). In cancer and BPH the tissue architecture is lost and the release of PSA into blood is increased (Bostwick, 1994). The serum concentrations of total PSA are often classified into three different ranges, 0-4 μg/L, 4-10 μg/L and >10 μg/L (Catalona et al., 1991). Serum levels < 4 μg/L are associated with low risk of prostate cancer, but the cut-off limit 4 μg/L does not take the effect of increasing prostate volume with age on serum PSA into account, and age-specific cut-off limits for PSA are also used (Oesterling, 1996) (Table 4). PSA levels ranging from 4 μg/L to 10 μg/L are commonly referred to as the grey zone, because elevated levels caused by BPH and PCa overlap. Patients with PSA levels > 10 μg/L have a likelihood of more than 50% of harboring prostate cancer (Catalona et al., 1993).

When reaching circulation, active PSA rapidly interacts with different protease inhibitors such as alpha$_1$-antichymotrypsin (ACT), alpha$_2$-macroglobulin (A2M) (Christensson et al., 1990) and alpha$_1$-proteinase inhibitor (API) (Zhang et al.,

Table 4. Age-specific reference limits for PSA

Age (years)	PSA (μg/L)
40-49	2.5
50-59	3.5
60-69	4.5
70-79	6.5

Reference: Oesterling et al. 1993

1997a). Most of the PSA in serum occurs in complex with ACT and minor part with A2M and API, whereas 5%-40% is free (Stenman et al., 1991; Lilja et al., 1991). Free PSA in serum has been found to consist of proPSA containing 2, 4 or 7 amino acids of the propeptide and mature, uncleaved free PSA as well as clipped form of free PSA termed BPSA, which is cleaved both after lysine 145 and lysine 182 (Mikolajczyk et al., 1997; Mikolajczyk et al., 2001; Mikolajczyk et al., 2000a; Mikolajczyk et al., 2000b; Wang et al., 2000). Assays for total serum PSA measure PSA-ACT, PSA-API and free PSA. The concentrations of PSA in serum are often increased by prostate cancer but moderately elevated levels are mostly caused by BPH. However, the proportion of PSA-ACT is higher and proportion of free PSA is lower in serum of prostate cancer patients than in men with BPH (Stenman et al., 1991; Lilja et al., 1991; Leinonen et al., 1993; Catalona et al., 1995). The proportion of PSA-API has also been found to be lower in men with cancer than in those with BPH (Zhang et al., 1999). The ratio of PSA-A2M to total PSA is also lower in prostate cancer than in BPH (Zhang et al., 2000). The proportion of the various proforms of free PSA is increased in cancer and this may improve the detection of aggressive prostate cancers in men with total PSA of 2-4 μg/L (Mikolajczyk et al., 2004; Catalona et al., 2004). The BPSA form of free PSA has recently been found to be a better predictor of BPH than either total PSA or free PSA (Canto et al., 2004) while PSA that is not nicked at lysine 145 has been shown to be more specific for prostate cancer (Nurmikko et al., 2001; Steuber et al., 2002)

Recently, it has been shown, that the hK2/free PSA ratio in serum has a better specificity without loss of sensitivity for PCa than total PSA or the free/total PSA ratio within the range of 4 to 10 μg/L of total PSA. Among men prescreened with PSA for prostate cancer, patients with high hK2 concentrations have a five- to eight-fold increased risk of prostate cancer. It has also been reported that hK2 significantly improves the identification of poorly differentiated (G3) tumors compared with PSA and is also helpful in the prediction of organ-confined disease (Kwiatkowski et al., 1998; Nam et al., 2000; Recker et al., 2000; Haese et al., 2005). However, these results have not been confirmed in other studies (Partin et al., 1999; Bangma et al., 2004).

1.3. PROSTATE SPECIFIC MEMBRANE ANTIGEN (PSM)

1.3.1. Gene, mRNA and protein

The gene encoding prostate specific membrane antigen (PSM/PSMA) also called folate hydrolase 1 (FOLH1) or glutamate carboxypeptidase II (GCP2), consists of 19 exons and it spans 62 kb of genomic DNA in chromosome regions 11p11.2 (Rinker-Schaeffer et al., 1995; Leek et al., 1995; O'Keefe et al., 1998; Maraj et al., 1998). The mRNA sequence of PSM is 2.65 kb and it encodes a 750-amino acid type II transmembrane glycoprotein of 100-kD (Troyer et al., 1995). An alternative mRNA splice variant of PSM mRNA, PSM', is generated from the mature transcript by use of an alternative splice donor site at position 114 in the PSM cDNA sequence (Su et al., 1995; O'Keefe et al., 1998). Translation of PSM' begins from a novel translation start codon producing a protein that lacks the first 57 amino acids coding for the intracellular and transmembrane domains. PSM' codes for a protein of 95 kD, which is localized to the cytoplasm (Su et al., 1995; Grauer et al., 1998). Two other splicing variants, PSM-C and PSM-D have also been found (O'Keefe et al., 2001). PSM-C begins transcription at the same nucleotide as PSM and PSM'. It uses the same splice donor site as PSM', but unique alternative splice acceptor sites located within intron one of the PSM gene. PSM-C

uses the same translation start codon as PSM' and would therefore produce a protein that is identical to PSM'. PSM-D has a new translation start codon within a new exon and 24 novel amino acids and the rest of the PSM gene protein in frame (O'Keefe et al., 2001). In chromosome 11q14, there is a highly conserved duplication of the PSM gene, *PSML* (PSMA-like), which possesses 98% identity to the *PSM* gene at the nucleotide level (Leek et al., 1995; O'Keefe et al., 2004).

1.3.2. Expression and function

PSM expression is highest in prostatic tissue. With the anti-PSM monoclonal antibody 7E11.C5 cytoplasmic immunoreactivity can be observed in benign epithelium, cancer and lymph node metastases. The expression increase from benign epithelium to high grade prostatic intraepithelial neoplasia (PIN) or adenocarcinoma, while the greatest extent and intensity is observed in the highest grades of cancer tissues. Furthermore, PSM expression is hormonally modulated by steroids and appears to be highest in hormone-deprived states (Horoszewicz et al., 1987; Israeli et al., 1994b; Bostwick et al., 1998; Sweat et al., 1998). PSM mRNA is down-regulated by androgens such as 5-α-dihydrotestosterone and up-regulated by growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor-alpha (TGF-α), and epidermal growth factor (EGF) (Israeli et al., 1994b; Kawakami et al., 1997; Wright et al., 1996). Although highly expressed in prostatic tissue, some other tissues also show PSM expression. By RNase protection assay expression has been found in the brain, salivary gland, and small intestine, but not muscle, kidney, liver or mammary gland (Israeli et al., 1994b). However, by sensitive nested RT-PCR, PSM mRNA expression has been found also in heart, liver, lung, spleen and kidney (Renneberg et al., 1999). Small amounts of PSM protein are additionally found in membranes and cytosols of breast, ovary and bone (Murphy et al., 2000). PSM mRNA and protein expression is also found in the endothelium of tumor-associated neovasculature of multiple nonprostatic solid malignancies (Liu et al., 1997; Silver et al., 1997; Chang et al., 1999b; Chang et al., 1999a). The *PSML* gene is expressed in kidney and liver under the control of a different promoter than the *PSM* gene, but no expression has been found in the prostatic tissue (O'Keefe et al., 2004).

Comparison of the expression of PSM mRNA in benign and malignant prostatic tissue shows 3 to 24-fold higher levels of PSM mRNA in prostatic cancer than in the normal prostate (Kawakami and Nakayama, 1997; Stamey et al., 2001; Burger et al., 2002; Schmittgen et al., 2003). In contrast, normal human prostate expresses more PSM' than PSM. BPH tissue shows similar expression of both variants. The ratio of PSM to PSM' mRNA has been shown to be lowest in the normal prostate and to increase with increasing Gleason score being greatest in hormone-refractory disease (Su et al., 1995; Schmittgen et al., 2003). The expression of the splicing variant PSM-C mRNA does not differ between normal prostate, prostate cancer and metastases. However, PSM-D mRNA has been found to be 2- to 5-fold higher in lymph node and bone metastases, respectively, when compared to primary prostate tumors. Expression is similar in normal prostatic tissue and primary prostate cancer (Schmittgen et al., 2003).

PSM is expressed in the prostate as a noncovalently associated homodimer (Schulke et al., 2003) and in the brain its extracellular domain exerts NAALADase activity cleaving the major neurotransmitter N-acetyl-aspartyl glutamate (NAAG) to yield N-acetyl-aspartate and free glutamate (Carter et al., 1996). PSM cDNA from human intestine has been cloned and both a full-length transcript and a 93 bp shorter transcript lacking exon 18 has been identified (Devlin et al., 2000). Intestinal

PSM has folate hydrolase activity cleaving glutamate residues from folate (Pinto et al., 1996). The role of the enzymatic activities of PSM in the prostate is still unclear.

1.3.3. Clinical aspects

PSM protein has been found in serum samples from prostatic cancer patients, in the seminal fluid of normal donors, in serum of BPH patients, and in normal male sera. PSM has also been found in sera of healthy females, and like in males, the levels increase with age, with the highest levels being found in sera from breast cancer patients (Rochon et al., 1994; Beckett et al., 1999). However, PSM levels in prostate cancer patients have been found by Western blotting and by protein biochip immunoassay to be significantly higher than in BPH patients or in normal controls (Rochon et al., 1994; Xiao et al., 2001).

Elevated PSM levels in prostate cancer have been found by enzyme-linked immunosorbent assay (ELISA) to predict clinical progression or resistance to therapy (Murphy et al., 1995). PSM has also been found to have prognostic significance, especially in patients with metastatic disease, and it correlates with the stage of disease (Murphy et al., 1996; Murphy et al., 1997).

In addition to Western blotting, ELISA and protein biochip immunoassay, PSM has been used in ProstaScint studies (Cytogen Corp., Princeton, NJ). ProstaScint utilizes the PSM antibody (7E11.C5), which labeled with the gamma-emitting ^{111}Indium has been used to image primary and metastatic foci of prostate carcinoma. This technique is approved by U.S. Food and Drug Administration (FDA) in the preoperative setting for patients with prostate cancer who are at high risk of metastatic disease and in patients with prostate cancer with a rising PSA level after prostatectomy (Sodee et al., 2000). Prostascint has been found to be more sensitive and accurate in detecting local or regional lymph node spread than CT, MRI and biopsy (Elgamal et al., 2000).

1.4. GENETIC CHANGES IN PROSTATE CANCER

The heterogeneity and multifocality of cancerous prostatic lesions, together with the relative small size of the prostate, make it difficult to obtain enough homogenous material for molecular analysis (Abate-Shen and Shen, 2000). Furthermore there are only a few prostate cancer cell lines suitable for in vitro studies, probably because of the slow growth of most prostate tumors (Berges et al., 1995). Additionally most commonly used cell lines LNCaP, PC3 and DU-145 have been isolated from metastatic lesions and not from primary tumors (Horoszewicz et al., 1983; Kaighn et al., 1979; Stone et al., 1978). Most studies in the literature are based on these cell lines, while the genetic information from primary prostatic tumors is still rather limited.

1.4.1. Germ line changes

The PSA gene has a polymorphic site in its ARE I at position –158 with two alleles, A and G. The GG genotype has been reported to be associated with more advanced disease at the time of diagnosis (Xue et al., 2000; Gsur et al., 2002). Together with a short glutamine repeat in the androgen receptor the GG genotype was found to be associated with significantly increased risk of malignant disease in younger men (<65 years) (Binnie et al., 2004). Slightly, but not statistically significantly lower PSA levels has been found in men with the AA genotype (Xu et al., 2002). Contradictory results showing homozygosity for the A-allele to be associated with higher serum PSA levels and with the presence of circulating tumor cells has been reported (Medeiros et al., 2002). Recently Wang et al. found no association between PSA ARE

I polymorphism and prostate cancer risk or serum PSA levels in Japanese men (Wang et al., 2003). However, genetic variations (single nucleotide polymorphism, SNP) in the PSA promoter (-4643G/A, -5412C/T, and -5429T/G) have been shown to be associated with increased serum levels in men without prostate cancer (Cramer et al., 2003).

Single nucleotide polymorphism at base 792C/T in the hK2 coding region has been found. 792C form encodes the active and 792T form encodes the inactive hK2 protein (Herrala et al., 1997). The inactive T allele is associated with lower serum hK2 levels, but a higher prostate cancer risk. This could be explained by lower baseline levels of hK2 both in normal and malignant prostate cells. Thus, increased hK2 production by prostate cancer cells would have a more pronounced association with prostate cancer risk for patients with low baseline production of hK2 (i.e., TT genotype) compared with patients who have high baseline hK2 levels (i.e., CC genotype) (Nam et al., 2003).

1.4.2. Somatic changes

At the chromosomal level losses of genetic material are more common than gains or amplifications in prostate cancer. The two most common regions with deletions are 8p and 13q (Porkka and Visakorpi, 2004). The most promising target gene for the 8p loss is a homeobox gene *NKX3.1* (He et al., 1997). Loss of protein expression of *NKX3.1* has been shown to correlate with tumor progression (Bowen et al., 2000). Although loss of the *NKX3.1* gene locus has been detected in prostate cancer, no mutations have been found in the remaining allele (Voeller et al., 1997). This may indicate haploinsufficiency as a mechanism abolishing the suppressive activity of NKX3.1. In one study increased expression of NKX3.1 mRNA has been found in prostate cancer tissue compared to normal tissue (Xu et al., 2000), which could indicate posttranscriptional control of *NKX3.1* gene expression.

The second most frequently deleted chromosomal region in prostate cancer is 13q. The retinoblastoma gene (*RB1*) at 13q14 is a strong candidate as a cancer suppressor gene in this area, but controversial results on the loss of the *RB1* locus have been reported (Latil et al., 1999; Chen et al., 2001; Ueda et al., 1999; Hosoki et al., 2002). Other regions showing losses in prostate cancer are 6q, 10q, 16q and 18q, indicating the presence of possible tumor suppressor genes in these regions (Porkka and Visakorpi, 2004).

PTEN gene on 10q23 has been found to be mutated in the prostate cancer cell lines LNCaP and DU-145 (Li et al., 1997). Deletions and mutations have also been reported more frequently in metastatic lesions of prostate cancer than in primary tumors (Dong et al., 2001; Vlietstra et al., 1998). Loss of heterozygosity (LOH) has been reported to be more frequent than mutations of *PTEN* in prostate cancer (Fernandez and Eng, 2002). Haploinsufficiency, where only one allele of *PTEN* is inactivated and the gene product from the remaining allele is insufficient to produce a normal phenotype, has been shown to promote prostate cancer progression (Kwabi-Addo et al., 2001).

The most common genomic change in prostate cancer is hypermethylation of the glutathione S-transferase (*GSTP1*) gene promoter, which is found in over 90% of prostate tumors and in 70% of PIN cases (Lee et al., 1994; Brooks et al., 1998).

Decreased expression of E-cadherin and mitogen-activated protein kinase kinase 4 (MKK4) proteins have been reported to be associated with prostate cancer metastasis (Umbas et al., 1992; Yoshida et al., 1999). E-cadherin expression has been found to be significantly lower in tumor tissues which were positive for PSA or PSM RT-PCR as compared to RT-PCR negative tissues (Mejean et al., 2000; Loric et al., 2001).

Expression of the cell cycle regulatory

genes *p27* and *p16* has been found to be altered in prostate cancer. P27 expression progressively decreases with increased tumor grade and stage (Tsihlias et al., 1998; Cote et al., 1998), while *P16* gene deletion and methylation combine to inactivate the gene in a subset of tumors and thus has been thought to represent a late event in prostate cancer progression (Jarrard et al., 1997).

Prostate stem cell antigen (PSCA) is a 123 amino acid protein first identified as an upregulated gene during cancer progression in the LAPC-4 prostate zenograft model. In the normal prostate, PSCA mRNA has been detected in a subset of basal and secretory cells (Reiter et al., 1998). It has been found to be overexpressed at the mRNA and protein levels in PCa and the expression has been found to correlate with increasing grade and stage of the tumor (Zhigang and Wenlv, 2004).

DD3 gene has been reported to be one of the most prostate cancer specific genes at present (Bussemakers et al., 1999). It is highly overexpressed in prostate cancer at mRNA level and it is regulated by a unique prostate cancer-specific transcriptional mechanism. However, no correlation between transcriptional activity and tumor stage or grade has been detected (de Kok et al., 2002).

When prostate cancer progresses from a hormone-sensitive, androgen-dependent stage to a hormone-refractory, androgen-independent tumor, the androgen receptor pathway functions despite anti-androgen therapy. The androgen receptor has been identified as a target gene for Xq amplification that is frequently found in hormone-refractory prostate tumors (Visakorpi et al., 1995; Linja et al., 2001). Polymorphism and mutations have also been found in the AR gene. These have been suggested to be associated with higher prostate cancer risk and antiandrogen treatment failure, respectively (Giovannucci et al., 1997; Taplin et al., 1995; Hara et al., 2003). It has also been shown that IGF-I, keratinocyte growth factor (KGF), EGF and HER-2/neu directly can activate the AR in the absence of androgens, which means that the androgen-signaling chain may be activated by growth factors in an androgen-depleted environment (Culig et al., 1994; Craft et al., 1999).

Increased mRNA levels of alpha-methylacyl-coenzyme A racemase (AMARC) in relation to PSA have been shown to be predictive of prostate cancer (Zielie et al., 2004) and immuno-histochemical staining for AMARC is now used in PCa diagnosis (Jiang et al., 2001; Rubin et al., 2002).

The Hedgehog signalling pathway, which also plays an essential role in developmental patterning, is required for regeneration of the prostatic epithelium (Karhadkar et al., 2004). Continuous pathway activation has been shown to transform prostate progenitor cells and render them tumorigenic. Furthermore, increased pathway activity has been found to distinguish metastatic from localized prostate cancer. Monitoring and manipulation of the Hedgehog pathway activity has been suggested to offer significant improvements in diagnosis and treatment of prostate cancer with metastatic potential (Karhadkar et al., 2004).

Recently, a new candidate tumor suppressor gene, *EPHB2* has been identified in prostate cancer by nonsense-mediated microarray analysis (Huusko et al., 2004). The DU 145 prostate cancer cell line has been found to carry a truncating mutation of *EPHB2* and a deletion of the remaining allele. Additional frameshift, splice site, missense and nonsense mutations were found in clinical prostate cancer samples. These findings suggest that inactivation of *EPHB2* may be important in the progression and metastasis of prostate cancer (Huusko et al., 2004).

Hepsin, a type II transmembrane serine protease, is found to be over-expressed in prostate cancer compared to normal prostatic tissue and BPH tissue both at the mRNA and protein levels (Luo et al.,

2001; Magee et al., 2001; Dhanasekaran et al., 2001; Stamey et al., 2001). The over-expression has been shown to cause disorganization of the basement membrane and it also promotes progression of the primary tumor and metastasis to liver, lung, and bone (Klezovitch et al., 2004). A significant correlation between the up-regulation of hepsin and tumor grade has been found, which suggests that hepsin could be a prognostic marker for tumor aggressiveness of PCa (Stephan et al., 2004).

1.5. RT-PCR TECHNIQUES

The polymerase chain reaction (PCR) was first proposed in the early 1970s by H. Ghobind Khorana and his colleagues as a strategy to lessen the labor involved in chemical synthesis of genes (Kleppe et al., 1971). However, at that time, when genes had not been sequenced and thermostable DNA polymerases had not been described, the idea was quickly forgotten. The technique was independently conceived 15 years later by Kary Mullis and coworkers, who described *in vitro* amplification of single-copy mammalian genes using the Klenow fragment of *Escherichia coli* DNA polymerase I (Saiki et al., 1985; Mullis et al., 1986; Mullis and Faloona, 1987). The use of a thermostable polymerase from *Thermus aquaticus* (Saiki et al., 1988) increased the efficiency of PCR and enabled automatization of the method.

Shortly after the introduction of PCR, Chelly et al. described the addition of another step to the PCR technique, i.e., the reverse transcription (RT), which made it possible to amplify segments of mRNA (Chelly et al., 1988). By RT-PCR, complementary DNA (cDNA) copies of RNA are amplified. The first step is the conversion of RNA to a single-stranded cDNA template by an RNA-dependent DNA polymerase. The extension of a cDNA copy starts from an oligonucleotide primer hybridized to RNA and it can then be amplified by PCR (Figure 1).

The advantages of RT-PCR over traditional Northern blot analysis are sensitivity, the need for only small quantities of RNA and a shortened analysis time. Additionally, RT-PCR can also provide more accurate quantitative information about mRNA levels. However, it is a complex technique and considerable attention should be given to its technical aspects.

1.5.1. RNA isolation

The quality of the RNA template in RT-PCR procedures is the most important determinant of reproducibility, especially in quantitative applications. It is therefore important to handle samples carefully at every stage, including collection, transporting and storage. RNA is extremely delicate once removed from its cellular environment and care should therefore be taken also in the purification step to avoid degradation. It has been shown that the amplification efficiency of different genes is affected differently by RNA degradation (Bustin and Nolan, 2004). Additionally, if mRNA is partially degraded, the cDNA yield is significantly lower near the 5´end of the mRNA than near the polyA tail (Swift et al., 2000).

Another important criterion for the RNA used in qRT-PCR is the absence of genomic DNA. DNA contamination causes inaccurate quantification of total RNA concentration and it can also inhibit the RT-PCR assay (Bustin and Nolan, 2004). There are also numerous components in blood and tissue that can inhibit RT-PCR assays, e.g, immunoglobulin G, hemoglobin and lactoferrin (Al-Soud et al., 2000; Al-Soud and Radstrom, 2001). DNAse-treatment prior to RT and testing RNA preparations for inhibitors increase the accuracy of quantification by RT-PCR (Bustin and Nolan, 2004).

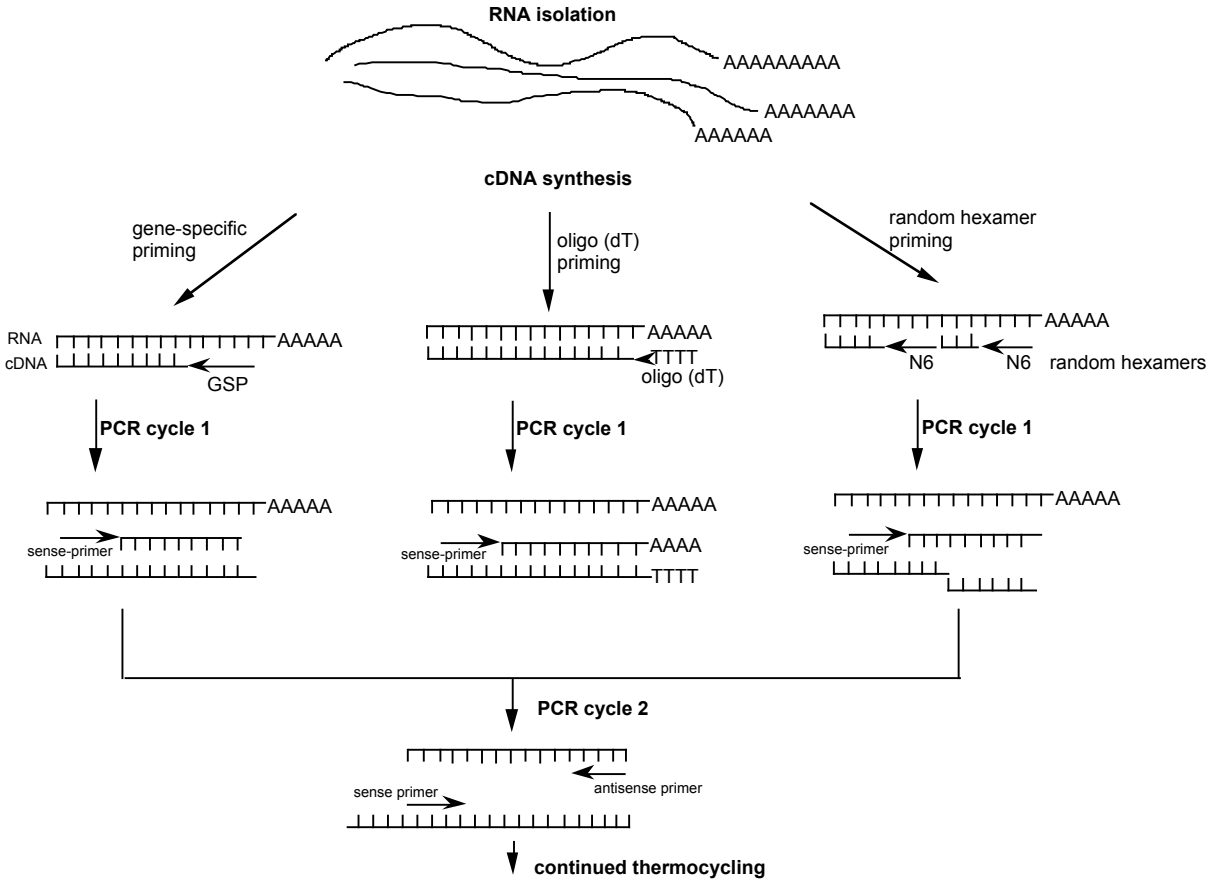

Figure 1. Schematic representation of various methods for amplification of RNA by PCR. GSP = gene specific primer.

1.5.2. Reverse transcription

In the qRT-PCR the RT step is critical for sensitive and accurate quantification and the amount of cDNA must accurately reflect the initial RNA content. It has been shown that experimental variation in RT-PCR is mainly attributable to the RT step. Reverse transcription efficiency depends on priming strategy, total RNA concentration and the enzyme used (Zhang and Byrne, 1999; Stahlberg et al., 2004a; Stahlberg et al., 2004b).

For the RT step specific primers, random primers or oligo-dT primers can be used (Figure 1). The advantage of oligo-dT primers and random primers is, that they maximize the number of mRNA molecules that can be analyzed from a small RNA sample. However, the majority of cDNA synthesized from total RNA by random primers is derived from ribosomal RNA (rRNA). This may create problems if the target mRNA is present at low levels. Oligo-dT primed cDNA synthesis is more specific as it does not transcribe rRNA. However, it will not prime RNA lacking the polyA tail or partly degraded RNA. Therefore it is unsuitable for RNA isolated from laser capture microdissected tissue or from archival material (Bustin and Nolan, 2004).

The use of target specific primers has been shown to be the most specific way to produce cDNA. Up to 24-fold differences have been measured by using gene specific primers compared to random hexamers (Stahlberg et al., 2004a). The main disadvantage of this method is that it requires different primers for each target.

Different reverse transcriptases produce different amounts of cDNA from a particular gene and up to 100-fold differences have been observed. The

concentration of total RNA and dithiotreitol (DTT) during cDNA synthesis has also been shown to be crucial for successful quantification of mRNA (Stahlberg et al., 2004a; Lekanne Deprez et al., 2002). Thus, for quantificating gene expression by reverse transcription, the same enzyme, same priming strategy and identical experimental conditions should be used.

1.5.3. Polymerase chain reaction

The polymerase chain reaction is dependent on the specificity of the oligonucleotide primers, the efficiency of the polymerase, and the experimental conditions, of which the primer specificity is most crucial. Mispriming and primer dimerization can signicantly affect the sensitivity of the PCR reaction and should be avoided (Chou et al., 1992). In RT-PCR, the primers should span exon-intron boundaries to avoid the amplification of genomic DNA.

A number of enzymes are available for PCR amplification. They vary in their fidelity, efficiency and ability to synthesize large DNA products. The most commonly used, Taq DNA polymerase, has 5'-3'-exonuclease activity but lacks 3'-5'-proofreading exonuclease activity making it efficient but somewhat inexact. Mixtures of two or more DNA polymerases can significantly increase the fidelity while maintaining the high efficiency of Taq (Cline et al., 1996). However, the proofreading activity can lead to primer degradation from the 3'-end and the rate of degradation is much faster for longer primers (de Noronha and Mullins, 1992).

The PCR reaction also needs deoxynucleotide triphosphates (dNTPs), divalent cations (usually Mg^{2+}) and monovalent cations (KCl) in a buffer, which maintains the right pH. Especially the concentrations of Mg^{2+} and dNTP should be strictly controlled. Mg^{2+} affects enzyme activity and increases the melting temperature (T_m) of double-stranded DNA. Imbalanced dNTP mixtures will reduce the polymerase fidelity and form soluble complexes with Mg^{2+} producing a substrate that the polymerase recoqnizes and amplifies (Eckert and Kunkel, 1991).

1.5.4. Detection and quantification of RT-PCR products

Qualitative detection of RT-PCR products is conventionally made by gel electrophoresis with ethidium bromide staining, but Southern blotting and/or sequencing of the product may be required to confirm the amplicon. Quantitative RT-PCR requires that the whole procedure is quantitative, i.e., isolation of the RNA, the RT and PCR steps and the detection of the RT-PCR products. Quantification also needs the use of control/reference genes or calibrators. The controls can be endogenous (e.g., housekeeping genes) or exogenous (e.g., synthetic DNA or RNA added to the sample). Both endogenous and exogenous controls can be either internal (amplified in the same tube) or external (amplified in separate tube). Different detection methods and controls used in qRT-PCR are discussed in following chapters.

1.5.4.1. Real-time quantification

Real-time PCR is a method that comprises both amplification and quantification of the PCR products simultaneously. Real-time PCR offers fast analysis of samples and the risk of post-amplification contamination of samples is abolished. The detection chemistries of all real-time PCR procedures are based on one of two principles for monitoring amplification products: binding of dyes to double-stranded DNA or hybridization of fluorescent probes to single-stranded DNA. Small molecules like SYBR Green bind to double-stranded DNA as minor groove binders (Morrison et al., 1998). Several approaches using target-specific hybridization to single-stranded DNA have been introduced, including hybridization probes (Wittwer et al., 1997),

TaqMan/hydrolysis probes (Holland et al., 1991; Livak et al., 1995), molecular beacons (Tyagi and Kramer, 1996) and scorpions (Nazarenko et al., 1997; Whitcombe et al., 1999) (Figure 2). The detection of the signal in these techniques is based on the fluorescence resonance energy transfer (FRET), which is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation energy is transferred from a donor molecule to an acceptor molecule.

Assays using two fluorescently labeled probes are based on one probe labeled with donor dye at the 3'-end and the other one labeled with an acceptor dye at 5'-end. When the amplicon is produced, the probes hybridize adjacent to each other within the amplified fragment and bring the two dyes next to each other allowing energy transfer between the dyes leading to a change in the fluorescent signal (Figure 2B).

Technologies using hydrolysis probes (for example Taqman), molecular beacons and scorpions utilize a hybridization probe with a reporter dye and a quencher dye at their 5'- and 3'-ends, respectively. In the Taqman technique, the probe is cleaved during PCR by the 5'-activity of DNA polymerase separating the dyes. Thus the quenching no longer occurs, which leads to a generation of a fluorescent signal (Figure 2C). When a molecular beacon hybridizes to its target sequence of the amplicon, the hairpin loop opens separating the dyes and generating a fluorescence signal (Figure 2D).

The fluorescence of DNA dyes or probes is monitored during each cycle of the PCR. At a certain point during amplification, the product accumulates to increase fluorescence above the background. This point is defined as the threshold cycle (C_t) and always occurs during the exponential phase of amplification. The relative copy number between two samples (experimental and control) can be determined by the difference in their respective C_t values (Higuchi et al., 1992; Gibson et al., 1996).

Methods using SYBR Green obviate the need for target-specific probes, but the specificity is determined entirely by the primers. As any double-stranded DNA generates fluorescence, additional specificity must be generated by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon (Ririe et al., 1997).

The use of target-specific probes increases the sensitivity of real-time PCR and enables multiplex approaches with differentially labeled fluorescent dyes.

1.5.4.2. Endpoint quantification

In addition to real-time quantification, several methods for quantification of endpoint PCR products have been described (Ikonen et al., 1992; Pannetier et al., 1993; Alard et al., 1993; Karttunen et al., 1996; Ylikoski et al., 1999). In endpoint quantification the PCR products are not measured during linear cycles, but after the reaction has reached a plateau phase. This is possible in the coamplification methods, where the amplification efficiencies of the templates are equal (Raeymaekers, 2000). Thus their ratio will remain constant despite the saturation of the reaction.

Minisequencing (single nucleotide primer extension) utilizes the specificity of DNA polymerase to incorporate a single nucleotide at the 3'-end of a sequence specific primer (Syvanen et al., 1990). During amplification, the PCR product is labeled using a biotinylated primer. Following amplification, the PCR product is captured on a streptavidin-coated solid phase and quantitatively detected by incorporating labeled nucleotides to a target-specific primer annealed immediately adjacent to the polymorphic site (Figure 3). Tritium has been used as a detection label in combination with scintillating microtiter plates (Ihalainen et al., 1994). Minisequencing has been used for quantification of rare mRNA transcripts

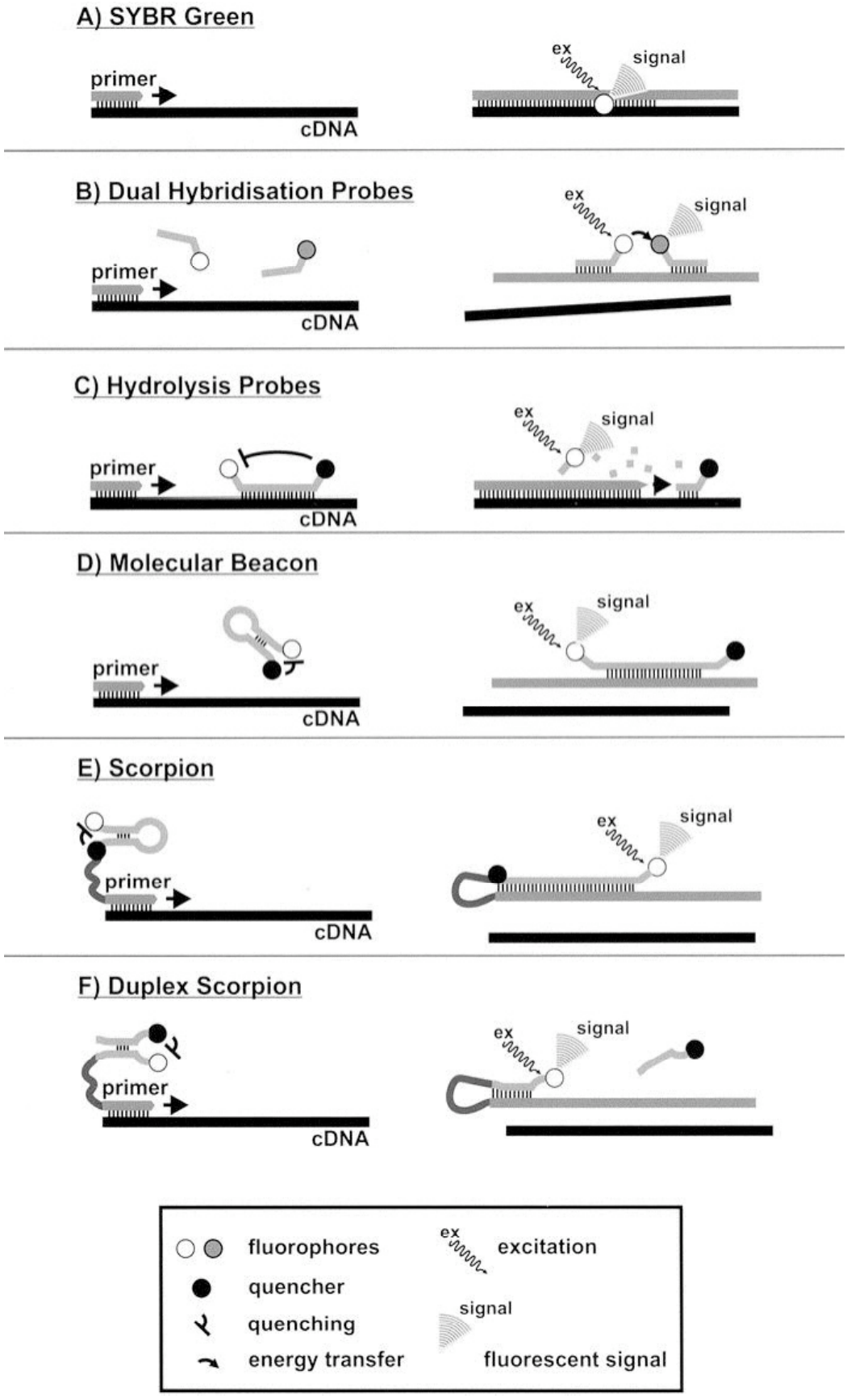

Figure 2. Different detection methods for real-time RT-PCR using specific intercalating agents like SYBR Green (A) or different techniques based on sequence specific labeled probes (B to F). From: Schrader et al. JUrol. vol. 169: 1858-64 (2003).

(Ikonen et al., 1992; Singer-Sam et al., 1992) and relative levels of two highly homologous transcripts (Karttunen et al., 1996; Stenman et al., 2001). When compared to assays using allele-specific probes (ASO), the power of discrimination between genotypes has been found to be one order of magnitude higher using the minisequencing method (Pastinen et al., 1997).

Time-resolved fluorometry has also been used for detection of quantitative RT-PCR products either as single-label or dual-label solution hybridization assays (Verhaegen et al., 1998; Ylikoski et al., 1999; Ylikoski et al., 2001a). Time-resolved fluorometry (TRF) with lantanide chelates is a very sensitive technique that utilizes the long decay time and the large Stoke's shift between excitation and emission wavelengths of the lantanide chelates (Syvanen et al., 1986).

Endpoint quantification for RT-PCR products has also been made by agarose gel electrophoresis or polyacrylamide gel electrophoresis and ethidium bromide staining followed by densitomety (Mularoni et al., 1996; Bor et al., 1998). In all endpoint quantification methods several steps are needed in contrast to real-time quantification, and extra care should be taken to avoid contamination during these processes.

1.5.5. Internal standards and normalization

RT-PCR can be “semi-quantitative” or quantitative containing either a reference gene for relative comparisons or a competitor for quantification. For relative comparisons endogenous housekeeping genes, supposed to be constitutively expressed and minimally regulated, have been used as reference genes. They have also been used widely as endogenous controls for quantitative real-time RT-PCR assays. However, the expression of the most commonly used housekeeping genes, e.g. GAPDH and β-actin, have been found to be highly variable (up to 30-70-fold) both between individuals and between biopses taken from the same patient (Tricarico et al., 2002; Dheda et al., 2004; Bas et al., 2004). Futhermore, GAPDH has been shown to be upregulated in human prostate carcinoma (Ripple and Wilding, 1995). Ribosomal RNAs which constitute 85-90 % of total cellular RNA have also been used as internal controls and they have been shown to be more reliable than the housekeeping genes (Zhong and Simons, 1999; Bas et al., 2004). However, rRNAs can not be used for normalization of mRNA samples and because of their high expression levels they are not suitable controls for genes with very low expression levels.

Alternatively, a synthesized exogenous DNA or RNA sharing identical PCR primer binding sites with target mRNA molecules can be used as competitors in qRT-PCR (Gilliland et al., 1990; Wang et al., 1989; Becker-Andre and Hahlbrock, 1989). An RNA competitor is added to cDNA synthesis and a DNA competitor can be added before or after the RT step. The advantage of using DNA is that DNA molecules are stable and easier to handle than RNA molecules. However, a DNA competitor measures only the efficiency of the PCR step in contrast to an RNA competitor, which also controls for the variation in the RT step. A DNA competitor has been shown to underestimate the number of target mRNA molecules by 10-fold when compared with an RNA competitor (Dufva et al., 1995). An even more accurate way is to add an RNA competitor to the sample before RNA extraction in order to control also for the extraction step (Ylikoski et al., 1999). The choice of the control molecule should be carefully considered to optimize the accuracy of each experiment.

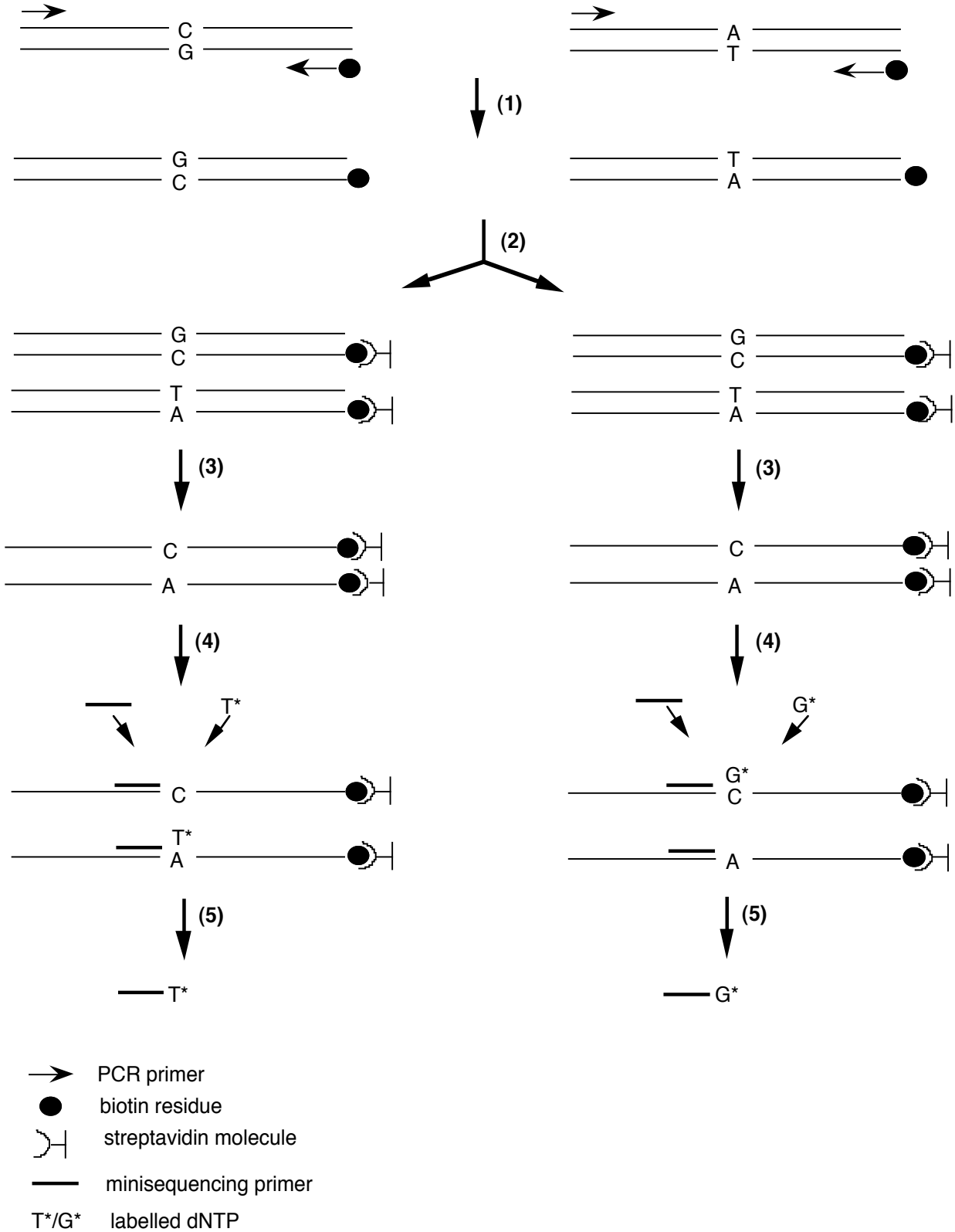

Figure 3. The minisequencing principle. (1) Biotinylation of DNA fragments during amplification using biotinylated primer. (2) Affinity-capture of two aliquots of amplified DNA fragments on a streptavidin-coated solid support. (3) Denaturation of the captured double stranded DNA fragments. (4) Identification of the variable nucleotides by annealing the minisequencing primer, which is extended with a single labeled dNTP. (5) Measurement of the incorporated label. Adapted from: Syvänen et al. Clin. Chim. Acta 226: 225-236 (1994).

1.6. HEMATOGENOUS SPREAD OF PROSTATE CANCER

The development of metastatic cancer is a complex process, in which malignant cells with invasive properties disseminate via blood or lymphatics from the primary tumor to the target organ. Once the invading cells penetrate into the lymphatic or vascular channels, they may grow there or a single cell or clumps of cells may detach and be transported within the circulatory system. The cells may then arrest at the capillary bed of the receptive organs, extravasate into the organ parenchyma, proliferate and establish a metastasis (Fidler, 2002). Animal experiments suggest that only about 0.01% of all circulating cancer cells actually form metastases (Liotta et al., 1974; Fidler and Hart, 1982).

Radical prostatectomy of prostate cancer is intended to provide complete cure, but up to 30%-50% of these cases recur, because prostate cancer cells from the primary tumor have escaped prior or during surgery to form metastases. Conventional macroscopic imaging techniques like bone scan and PSA are of limited value for identification of these cases and improved or new diagnostic and prognostic modalities are needed to reliably identify patients who have the best chance of benefiting from surgical treatment.

Circulating cancer cells can be identified in circulation of a patient by certain methods. Immunocytological methods have successfully been used in several studies to identify tumor cells in peripheral blood (Moss and Sanders, 1990; Hamdy et al., 1992) bone marrow (Redding et al., 1983; Pantel et al., 1996) and lymph node (Trojani et al., 1987). Immunocytological studies for detection of bone marrow micrometastases have demonstrated an increased risk for recurrent cancer of the breast, lung, stomach and colon (Pelkey et al., 1996). However, this method is dependent on the availability of specific antibodies to cell-surface antigens.

Since 1991, when Smith and coworkers first reported the detection of melanoma cells in circulation by RT-PCR (Smith et al., 1991), several studies have been performed on different cancers. Primers for the genes of interest are possible to design according the cDNA sequence and RT-PCR is sensitive enough for detection of rare cancer cells in circulation.

Several qualitative RT-PCR studies of the expression of PSA mRNA in circulation of prostate cancer patients have been done during last ten years. The clinical sensitivity of the method (percentage of patients with metastases who tested positive using RT-PCR for PSA) for peripheral blood averages 51% (Table 5). In contrast, when bone marrow was used as tissue source it averages 78% (Corey et al., 1997b; Wood et al., 1994; Deguchi et al., 1997; Melchior et al., 1997). The false positive rate for assay of PSA mRNA ranges from 0% to 40% (Table 5). For PSM the clinical sensitivity averages 80% but at the same time the positive false rate increases to 0%-96% (Table 6). The detection limit in most RT-PCR assays for PSA and PSM is 1 LNCaP cell equivalent/10^6 blood mononuclear blood cells regardless of the number of PCR cycles, which in these studies rarely exceeds 70 cycles. However, when nested RT-PCR with total of 80 cycles was used, the false positive rate was increased to 40%-100% (Henke et al., 1997; Smith et al., 1995; McIntyre et al., 2000). A possible explanation for the high false positive rates is illegitimate transcripts detected by extremely sensitive assays in normal blood cells (Chelly et al., 1991).

The first studies which measured PSA mRNA by RT-PCR in circulation used autoradiography to measure the radio-actively labeled PCR product or densitometry to measure ethidium bromide intensity (Sokoloff et al., 1996; O'Hara et al., 1996). In these studies beta-actin was used as a refence gene. A semiquantitative

assay with an internal DNA control was described in 1997, but no patient samples were studied (Corey et al., 1997a). The first quantitative studies for detection of PSA mRNA used an internal RNA control and time–resolved fluorometry (Verhaegen et al., 1998; Ylikoski et al., 1999). An internal RNA control has usually been used in quantitative RT-PCR studies for PSA (Verhaegen et al., 1998; Ylikoski et al., 1999; Ylikoski et al., 2001b; Ylikoski et al., 2002; Emmanouilidou et al., 2003; Kurek et al., 2003). In real-time RT-PCR studies, the quantification has been achieved with an external calibration curve generated with LNCaP cells or RNA isolated from LNCaP cells (Gelmini et al., 2001; Straub et al., 2001; Straub et al., 2003). Comparison of different qRT-PCR assays for PSA is difficult, because they all have used different procedures. From these studies, however, it can be concluded, that the amount of PSA mRNA copies is higher in blood of patients with metastatic PCa than with organ confined disease. Although PSA mRNA molecules has been found also in control samples, the levels are lower than in PCa patient samples (Ylikoski et al., 1999; Ylikoski et al., 2001b; Straub et al., 2001; Ylikoski et al., 2002; Emmanouilidou et al., 2003).

RT-PCR has also been used to study the effect of radical prostatectomy on the levels of PSA mRNA in circulation. The frequency of patients with CTCs and local PCa was found to increase after radical prostatectomy in 4 studies, but to decrease in two other ones (Oefelein et al., 1996b; Ogawa et al., 1999; Oefelein et al., 1999; Okegawa et al., 2000; Straub et al., 2001; Shariat et al., 2003b). The number of patients with advanced cancer and CTCs in their circulation was found to be unchanged after radical prostatectomy in one and to increase in another study (Okegawa et al., 2000; Straub et al., 2001). It has been speculated that radical prostatectomy may increase the risk of hematogenous dissemination of PCa (Ogawa et al., 1999) and that post-operative PSA mRNA positivity could be a predictor of disease progression (Shariat et al., 2003a). However, no correlation between post-operative PSA positivity after RP and Gleason score or stage of the disease in several studies has been found (Oefelein et al., 1996a; de Cremoux et al., 1997; Mejean et al., 2000; Thomas et al., 2002; Shariat et al., 2002). Longer follow-up and standardized techniques are needed to evaluate whether RT-PCR methods play a role in “molecular staging” of prostate cancer.

Table 5. Methods, sensitivities and specificities of qualitative RT-PCR for PSA mRNA.

Reference	Method	Sensitivity	PSA positivity (A)	PSA positivity (B)	PSA positivity (C)	PSA positivity (D)
Moreno et al., 1992	RT-PCR, AGE, Sequencing	ND	ND	ND	33% (4/12)	0% (0/17)
Katz et al., 1994	RT-PCR, AGE, Southern analysis, Chemilumin.detection.	1 LNCaP/10e5 BMC	39% (25/65)	ND	78% (14/18)	0% (0/65)
Seid en et al., 1994	RT-nested PCR, AGE, Southern analysis	10 LNCaP /mL blood	6% (6/100)	ND	31% (11/35)	0% (0/14)
Katz et al., 1995	RT-PCR, AGE, Southern analysis, Chemilumin.detection	1 LNCaP /10e5 BMC	35% (33/95)	ND	75% (6/8)	ND
Cama et a l., 1995	RT-PCR, AGE, Southern analysis, Chemilumin.detection	ND	34% (27/80)	ND	83% (5/6)	0% (0/65)
Ghossein et al., 1995	RT-PCR, AGE, Southern analysis	1 LNCaP /10e6 BMC	24% (6/25)	ND	34% (28/82)	0% (0/27)
Galvan et al., 1995	RT-PCR, microtiter based TRF	1 LNCaP /10e6 BMC	not tested	ND	ND	ND
Fadlon et al., 1996	RT-PCR, AGE, Southern analysis	ND	0% (0/2)	ND	10% (1/10)	0% (0/5)
Brandt et al., 1996	RT-nested PCR, AGE	10pg to 1 ng	ND	ND	6% (6/10)	0% (0/4 BPH)
Zhang et al., 1997b	RT-nested PCR, AGE	1 LNCaP /10e6 BMC	13% (6/48)	ND	64% (7/11)	0% (0/20)
Corey et al., 1997b	RT-PCR, AGE	1 LNCaP /10e8 BMC	19% (12/63)	ND	46% (6/13)	0% (0/20)
Thiounn et al., 1997	RT-PCR, AGE, Southern analysis	1 LNCaP /10e6 BMC	22% (10/46)	ND	ND	11% (17/145)
Castaldo et al., 1997	RT-PCR, AGE, Dot plot	1 LNCaP /10e6 BMC	23% (4/17)	ND	50% (3/6)	0% (8/8)
Ign atoff et al., 1997	RT-PCR, AGE, radioactive PAGE	ND	21% (17/82)	ND	100% (11/11)	0% (0/6)
Gao et al., 1999	RT-nested PCR, AGE	1 LNCaP /10e7 BMC	32% (27/85)	42% (5/12)	ND	0% (0/22)
Millon et al., 1999	RT-PCR, AGE	ND	13% (4/31)	ND	ND	ND
McIntyre et al., 2000	RT-nested PCR (40+40 cycles) , AGE	2 LNCaP /mL blood	ND	ND	ND	40%(4/10)
McIntyre et al., 2000	RT-nested PCR (25+25 cycles), AGE	4 LNCaP /mL blood	ND	ND	ND	0% (0/10)
Hara et al., 2002	RT-nested PCR, AGE	1 LNCaP /mL blood	2% (1/41)	0% (0/2)	50% (7/14)	2% (1/71)
Gao et al., 2003	Epithelial cell enrichment ,RT-nested PCR, PAGE, SYBR Gold	1 LNCaP /mL blood	80% (108/135)	ND	ND	2% (1/45)
Schamhart et al., 2003	RT-nested PCR, AGE	1 LNCaP /mL blood	34% (14/34)	ND	ND	ND
Kurek et al., 2004	RT-nested PCR, AGE, Southern analysis	1 LNCaP /10e6 BMC	32% (76/238)	40% (48/120)	ND	ND

ND= not determined
(A)= blood samples from patients with local tumors
(B)= blood samples from patients with advanced tumors
(C)= blood samples from patients with metastatic tumors
(D)=blood samples from controls
AGE=agarose gel electrophoresis
PAGE=polyacrylamide gel electrophoresis
TRF=time resolved fluorescence
BPH=benign prostatic hyperplasia

Table 6. Methods, sensitivities and specificities of RT-PCR studies for PSM.

Reference	Method	Detection limit or linear range	PSM mRNA positivity (A)	PSM mRNA positivity (B)	PSM mRNA positivity (C)
Israeli et al., 1994	RT-nested PCR	1 LNCaP/10e6 MCF-7 cells	72%(13/18)	60% (9/15)	10% (4/39)
Israeli et al., 1995	RT-nested PCR	1 LNCaP/10e6 MCF-7 cells	63% (19/30)	ND	0% (0/16)
Loric et al., 1995	RT-nested PCR	1 LNCaP/10e6 BMC	44% (12/27)	85% (28/33)	0% (0/35), 0% (0/18 BPH)
Eschwege et al., 1995	RT-nested PCR	ND	60% (3/5)	ND	0% (0/6)
Noguchi et al., 1997	RT-nested PCR	ND	55% (38/69	ND	0% (0/40)
Zhang et al., 1997b	RT-nested PCR	1 LNCaP/10e6 BMC	16% (4/25)	91% (10/11)	0% (0/11), 0% (0/9 BPH)
Gala et al., 1998	RT-nested PCR	2 LNCaP/10e6 BMC		ND	96% (24/25)
Grasso et al., 1998	RT-nested PCR	ND	43% (46/107)	91% (10/11)	0% (0/20), 0% (0/15 BPH)
Lucotte et al., 1998	RT-nested PCR	1 LNCaP/10e6 BMC	64% (16/25)	ND	0% (0/35), 0% (0/15 BPH)
Renneberg et al., 1999	RT-nested PCR	ND	ND	ND	ND
Saimoto et al., 1999	RT-nested PCR	ND	85% (6/7)	ND	0% (0/6), 75% (3/4 BPH)
Okegawa et al., 1999	RT-nested PCR	ND	pT2: 18% (3/17)	pT3: 64% (9/14), N1: 89% (8/9)	ND
Millon et al., 1999	RT-nested PCR	ND	79% (38/48)	ND	71% (10/14)
McIntyre et al., 2000	RT-nested PCR	4 LNCaP /mL blood	19% (5/26)	ND	0 %
Loric et al., 2001	RT-nested PCR	ND	55% (24/44)	ND	ND
Llanes et al., 2002	RT-nested PCR	ND	ND	ND	38% (23/60)
Hara et al., 2002	RT-nested PCR	1 LNCaP/mL blood	21% (12/58)	ND	3% (2/71)
Thomas et al., 2002	RT-nested PCR	ND	69%	ND	ND
Nagao et al., 2002	RT-nested PCR	ND	ND	ND	ND
Adsan et al., 2002	RT-nested PCR	1LNCaP/10e6 MS cell	pT1: 0%, pT2: 37%	pT3: 42%, N1:48%	0% (0/20)
Hisatomi et al., 2002	RT-nested PCR	ND	ND	ND	PSM: 12.5%, PSM': 29%
Varkarakis et al., 2003	RT-nested PCR	1 LNCaP/10e6 BMC	22%	pT3: 60%, T4: 95%	0% (0/20)
Chu et al., 2004	RT-nested PCR vs. Real-time PCR	0.0005 ng vs. 0.5 ng	59% (41/70) vs. 27% (19/70)	ND	53% (10/19) vs. 4% (8/199)
(Emmanouilidou et al., 2004)	RT-PCR, Solution hyhridization, Chemiluminescence	500-5x 106 PSM copies	ND	ND	ND

A= blood samples from patients with local tumors
B= blood samples from patients with advanced or metastaic rumors
C= blood samples from controls

2. AIMS OF THE STUDY

The aims of this study were:

1. To develop an RT-PCR method to detect PSA mRNA-expressing cells in circulation of prostate cancer patients.

2. To develop an RT-PCR method to detect PSM mRNA-expressing cells in circulation of prostate cancer patients.

3. To develop a quantitative method to measure the expression levels of PSA mRNA in blood of cancer patients and healthy individuals and to compare the expression levels of PSA mRNA at different time points before and after prostatic surgery and endocrine treatment of PCa.

4. To develop a quantitative method to measure the relative expression levels of PSA and hK2 mRNAs in benign and malignant prostatic tissue.

3. MATERIALS AND METHODS

3.1. SAMPLES

3.1.1. Cultured cells

LNCaP and DU-145 cell lines were obtained from the American Type Culture Collection. LNCaP cells were used for cell spiking experiments to estimate the copy number of PSA mRNA per cell (I, II) and to evaluate the reproducibility of the quantitative RT-PCR (III, IV). DU-145 cells were used as a negative control for PSA and hK2 mRNA expression (IV). Cells were cultured in RPMI 1640 medium supplemented with 100 IU/mL penicillin, 0.1 μg/mL streptomycin (HyClone Europe Ltd, Cramlington, UK), 10% fetal calf serum (Flow Laboratories, UK), 2 mM glutamine and 2.5 μg/mL amphotericin B (GibcoBRL; Paisley, Scotland).

3.1.2. Blood samples

Blood samples from 55 patients with PCa, 29 with BPH, two with renal stones, and three with other cancers (one renal cancer, one testicular cancer, and one bladder cancer) were obtained from the Department of Urology, Helsinki University Central Hospital. Blood samples from males less than 40 years of age and from females were obtained from the Hematology Laboratory of Helsinki University Central Hospital (n=42 and n=46, respectively) and from healthy laboratory personnel (7 males and 6 females) (I, II, III). A single sample was obtained from 25 PCa patients after surgical therapy and from 4 BPH patients, two patients with renal stones, and three with other cancers and used in studies I and II.

From 2 to 10 blood samples were collected before, during and from 1 h to 6 months after biopsy or surgery from 30 PCa and from 25 BPH patients. These patients included 15 men undergoing needle biopsy, 17 treated by transurethral resection of the prostate (TURP) because of benign prostatic hyperplasia (BPH), 15 men treated by radical prostatectomy and 8 by orchiectomy. One patient receiving total androgen blockade was also studied (III).

3.1.3. Tissue samples

Tissue samples of malignant prostate (PCa) (n=13), normal prostate (n=3) and hyperplastic prostate (n=14) were collected at the Department of Urology, Malmö University Hospital, Sweden and at the Department of Pathology, HUCH (IV). Two pairs of cancerous/benign tissue samples were collected during radical prostatectomy. Twelve PCa samples, one normal prostate tissue and all BPH samples were collected during TURP. Total RNA and cDNA from normal prostatic tissue and cDNA from the prostatic adenocarcinoma cell line (PC-3) were obtained from Clontech (Palo Alto, CA).

3.2. DETECTION AND QUANTIFICATION OF THE mRNA

3.2.1. RNA extraction

Blood samples (I, II, III) were drawn into 10 mL Vacutainer EDTA tubes (Becton Dickinson, Rutherford, NJ). Erythrocytes were hemolyzed within 2 h of venipuncture by incubating 1 volume of blood with 1.5 volumes of diethylpyrocarbonate-treated water for 5 minutes before collection of the nucleated cells by centrifugation at 1200 x g for 10 min. Total RNA was extracted from nucleated cells, from exponentially growing cultured cells and from fresh tissue specimens according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi,

1987) or with Phase Lock Gel (5 Prime -->3 Prime, Boulder, CO). Total RNA integrity was checked by formaldehyde gel electrophoresis. In addition, RT-PCR with specific β-globin and β-actin control primers were used for blood and tissue samples, respectively.

3.2.2. Determination of the detection limit

The detection limit of the qualitative RT-PCR method was established by spiking blood samples with LNCaP cells, a prostate carcinoma cell line expressing PSA. LNCaP cells were cultured in RPMI-1640 medium as described earlier. The leukocytes in the blood samples and the LNCaP cells were counted and dilutions of LNCaP cells were mixed with blood to give 1-1000 LNCaP cells per 6 x 10^6 leukocytes/mL. Total RNA was isolated and RT-PCR was done as described above.

3.2.3. Oligonucleotide primers

Oligonucleotide primers used for RT-PCR of PSA, PSM and hK2 and for quantitative detection of PSA and hK2 are shown in Table 7. Primers for RT-PCR were chosen from different exons to avoid amplification of genomic DNA. The quality of the isolated RNA was verified by control RT-PCRs using β-globin or β-actin primers. The sequences for the β-globin primers were: 5'-ACC CAG AGG TTC TTT GAC TC-3'(sense) and 5'-TCT GAT AGG CAG CCT GCA CT-3'(antisense), and for β-actin: 5'-CCC AGG CAC CAG GGC GTG AT-3'(sense) and 5'-TCA AAC ATG ATC TGG GTC AT-3'(antisense).

3.2.4. Internal standards

In the quantitative RT-PCR, mutated PSA RNA (III) or cDNA as internal standard (IS) (IV) were used. A mutated PSA cDNA with a two-nucleotide deletion (nucleotides 600 and 601, GenBank

Table 7. PSA, hK2 and PSM primers used in studies I-IV.

Primer	Sequence	nt	Exon (#)	minisequencing ^{3}H-nt	used in
PSA outer sense	CACAGGCCAGGTATTTCAGG	296-315	3		I, III
PSA outer antisense/ cDNA	CCTTGATCCACTTCCGGTAA	785-804	5		I, III
PSA nested sense	TCCAATGACGTGTGTGCGCA	579-598	4		I, III *
PSA nested antisense	GTGTACAGGGAAGGCCTTTC	753-772	5		I, III
PSA minisequencing	GGTCACCTTCTGAGGGTGAAC	600-620		*dTTp	III
IS minisequencing	TTGGTCACCTTCTGAGGGTGA			*dTTP	III
PSM outer sense	CAGATATGTCATTCTGGGAGGTC	1266-1288	10		II
PSM outer antisense/ cDNA	AACACCATCCCTCCTCGAACC	1893-1913	16		II
PSM nested sense	CCTAACAAAAGAGCTGAAAAGCCC	1587-1610	13/14		II
PSM nested antisense	ACTGTGATACAGTGGATAGCCGCT	1798-1821	16		II
PSA/ hK2 cDNA	GATGGTGTCCTTGATCCACT	793-812	5		IV
PSA/ hK2 sense	CACTGGGGACCACCTGCTA	481-499	3		IV
PSA/ hK2 antisense	bio-CACTTCCGGTAATGCACCAC	777-796	5		IV
PSA/ IS minisequencing	AGAAACTTCAGTGTGTGG	547-564		*dATP/*dTTP	IV
hK2 minisequencing	AGGAGTCTTCAGTGTGTG	546-563		*dATP	IV

Accession numbers of PSA, PSM and hK2 are: NM_001648.2, BC025672 and BC005196.1.
* primer was 5´-biotinylated in study III

NM_001648.2) in the vector pGEM-3 was kindly provided by Dr. Alice Ylikoski, Department of Biotechnology, University of Turku. The plasmid was linearized and used for RNA synthesis with the AmpliScribe T7-kit (Epicentre Technologies Corporation, Madison, Wisconsin). The RNA was purified with Sephadex G-50 (Pharmacia NICK-Columns, Pharmacia Biotech, Uppsala, Sweden) and used as an internal RNA standard (III).

For preparation of the internal cDNA standard (IV), a substitution of A to T was made in nucleotide 565 of PSA cDNA (GenBank NM_001648.2) by site directed mutagenesis (Ho et al., 1989). Mutated PSA cDNA was cloned from nucleotide 481 to 812 into a pCR II plasmid vector (Invitrogen).

3.2.5. RT-PCR

3.2.5.1. Qualitative assays

Nested qualitative RT-PCR assays were used for PSA mRNA (I) and PSM mRNA (II). One μg of total RNA was transcribed into cDNA using the gene-specific antisense PCR primer (Table 7) and AMV (Promega, Madison, WI) or M-MLV Reverse Transcriptase (GibcoBRL, Gaithersburg, MD) according to manufacturer's instructions. The possible contamination of the RNA samples with cDNA was excluded by subjecting each sample to the RT-reaction without RT-enzyme before PCR. For PCR, five μl of 1/50 diluted cDNA (I) or 2 μl cDNA (II) was amplified with the outer primers (Table 7) in a 50-μl reaction volume. For reamplification, five μl (I) or 2 μl (II) of the first PCR product was further amplified with the nested primers (Table 7) in a 50 μl reaction volume. The amplification conditions for PSA and PSM mRNA are described in publications I and II, respectively. The RT-PCR products were separated in 1.5 % agarose gel and stained with ethidium bromide. RNA isolated from prostatic tissue was used as a positive control and water as a negative control in all experiments.

3.2.5.2. Quantitative assays

Total RNA from 1 mL of blood and 100 molecules of IS RNA (III) or 1 μg of total RNA and 10^4 molecules of IS cDNA (IV) were reverse transcribed with SuperScriptII RNase H^- Reverse Transcriptase (GibcoBRL Life Technologies) using the gene-specific antisense primer (Table 7). For the PCR reaction, one tenth (2 μl) of cDNA with outer primers (Table 7) in a 50-μl reaction volume following the amplification with the nested primers (biotinylated sense and antisense, twenty pmol and 100 pmol, respectively) in a 75-μl reaction volume (III) or 30% of cDNA using 80 pmol of sense primer and 16 pmol of biotinylated antisense primer (Table 7) in an 80-μl reaction volume (IV), was amplified as described in III and IV. Ten μl of the product was electrophoresed in a 2 % agarose gel and visualized by ethidium bromide staining.

3.2.6. Restriction enzyme analysis, cloning and sequencing of the PCR products

In qualitative assays, the identity of the PCR products was confirmed with Ava I (I) or BstXI (II) restriction enzyme digestion and sequencing of the product. These enzymes have only one restriction site within the PSA and PSM PCR products, respectively. For sequencing, the PCR products were cloned into a pCRII plasmid vector (TA Cloning version 1.3; Invitrogen). Plasmid DNA was sequenced with Version 2.0 DNA Sequencing Kit (United States Biochemical).

3.2.7. Southern blot analysis

1.5 % agarose gel containing 16 negative samples and one positive control sample was blotted onto Hybond-N (Amersham Intl, Amersham, UK) nylon membrane according to the manufacturer´ s instructions. The hybridization was carried out at 65° C overnight in hybridization solution containing 1 mol/L NaCl, 10% dextran sulphate and 1% SDS. The membrane was washed under high stringency conditions. The sequenced PCR-product, labeled by nick translation, was used as a probe (I).

3.2.8. Solid-phase minisequencing

For quantification of PCR products the biotinylated PCR product was captured on a streptavidin coated scintillating microtitration plate (ScintiPlate, Wallac). The bound DNA fragment was denaturated with 50 mM NaOH leaving the biotinylated antisense strand bound to the well. Primer annealing and extension were carried out in a minisequencing reaction mixture containing the detection primer and the appropriate ^{3}H-labeled nucleotide (Amersham) shown in Table 7. PCR products from wild type and from IS were detected in separate reactions. The radioactivity was measured in a scintillation counter. For each sample, the ratio between the incorporated radioactive nucleotides (PSA/IS or PSA/ hK2, in III and IV, respectively) was calculated.

3.3. IMMUNOFLUOROMETRIC ASSAY FOR PSA

The DELFIA Prostatus PSA free/total kit (Wallac), which simultaneously detects free PSA and total PSA was used to measure total PSA in plasma samples collected at different time points before and after prostatic operations (III).

3.4. STATISTICAL METHODS

The difference in serum PSA concentrations between patients who tested positive or negative for PSA mRNA by qualitative RT-PCR assay was analyzed by Student's t-test after logarithmic transformation of the PSA values (I). The difference in pre-or postoperative serum PSA level, biopsy grade or Gleason score, tumor grade or disease stage between PSA mRNA positive and negative groups was estimated by using the Mann-Whitney U-test (III). The statistical significance of the differences between PSA/hK2 mRNA ratios (IV) in various sample groups was calculated using the Mann-Whitney U-test (IV).

4. RESULTS AND DISCUSSION

4.1. RT-PCR ASSAYS FOR PSA, hK2 AND PSM mRNA

4.1.1. Qualitative assays

Qualitative RT-PCR assays were used for detecting PSA- or PSM-expressing cells in circulation of prostate cancer patients (I, II). To determine the detection limit of the assays, LNCaP cells were diluted into blood sample from a healthy female donor. PSA mRNA was detected by 30+30 cycles of RT-PCR in samples containing 10-1000 LNCaP cells/mL of blood. Thus LNCaP cells could be detected at a concentration of 1.6 cells/10^6 blood leukocytes. This corresponds to the sensitivity obtained in other studies (Seiden et al., 1994; Ghossein et al., 1995; Thiounn et al., 1997; Castaldo et al., 1997).

All the dilutions containing 1-1000 LNCaP cells/mL of blood were positive for PSM mRNA by 25+25 cycles of RT-PCR. Thus the sensitivity of this method is below 1 LNCaP cell/mL. All controls without RT-enzyme were negative as well as control samples without template. All the negative patient samples were also negative by Southern blot analysis, which has a detection limit below that of ethidium bromide staining. Total RNA integrity was tested by control RT-PCR reactions, in which all samples were positive for β-globin or β-actin (publication II, Figure 1). Contamination of RNA samples with prostatic RNA was excluded by RT-PCR with PSA primers before PSM mRNA RT-PCR (publication II, Figure 2). The identity of the PCR products was confirmed by restriction enzyme analysis and sequencing of the PCR product. The products had the expected sequences of PSA or PSM.

4.1.2. Quantitative assays

Two different quantitative RT-PCR assays for PSA mRNA were used to measure the absolute amounts of PSA mRNA molecules or the relative levels of PSA and hK2 mRNA molecules in blood or tissue samples (III, IV). In the first assay (III), where absolute amounts of PSA were measured, the primers amplified wild type; GenBank Accession No NM_001648 (Lundwall and Lilja, 1987) and 5 out of the 13 known splicing variants of PSA mRNA, which are M21896 (Riegman et al., 1988), U17040 (Monne et al., 1994), PSA-RP1 (Heuze et al., 1999), AJ459782 and AJ459783 (Heuze-Vourc'h et al., 2003). Eight out of 13 splicing variants are not amplified by these primers; AJ310937/M21897 and AJ310938 (Heuze-Vourc'h et al., 2001), AJ512346 (Heuze-Vourc'h et al., 2003), AF335477 and AF335478 (David et al., 2002), alternative spliced PSA by Tanaka et al. (Tanaka et al., 2000), NM_145864 (Lundwall and Lilja, 1987) and AJ459784 (Heuze-Vourc'h et al., 2003). From M21896 and PSA-RP1 a 636 bp transcript is produced, but only transcripts of 194 bp, produced from wild type and other variants were detected by agarose gel electrophoresis indicating absence or low expression levels of the longer PSA mRNAs.

The internal standard used in the quantitative PCR reaction competes with the target analyte and thus it influences the sensitivity of the method. The detection limit of the first assay as defined by the mean + 2 SD in negative samples was 7 copies of PSA mRNA when 100 molecules of the internal standard were used. In earlier studies different relationships between IS and detection limit have been observed, i.e. 50 copies PSA mRNA with 5000 copies of IS RNA (Ylikoski et al., 2002) and 8 copies of squamous cell carcinoma antigen mRNA with 20 copies

plasmid DNA (Stenman et al., 2003). Amplification of PSA mRNA was observed in 42% of the 88 control samples, and the highest concentration observed was 25 copies/mL blood. Therefore the cutoff level was defined as 26 copies/mL blood and thus all control samples were negative. The standard curve was linear from 25 to 1000 copies when the number of copies was plotted against the ratio of PSA mRNA and IS RNA (Figure 4). The reproducibility of the assay was evaluated with RNA in dilutions corresponding to 1, 10, 100, and 1000 LNCaP cells per 10^7 blood leukocytes in 5 separate RT-PCR assays. The interassay coefficient of variation (CV%) in these samples was 26%, 29%, 13% and 27%, respectively. The mean number (±SD) of PSA mRNA molecules in one LNCaP cell was calculated to be 887 (±393).

To measure the absolute concentrations of PSA mRNA, different mRNA or DNA standards have been used. Housekeeping genes like beta-actin or GAPDH require different primers than the target gene, and thus causes differences in amplification efficiency (Sokoloff et al., 1996; O'Hara et al., 1996). DNA standards with a length difference like a small insertion eliminate this problem (Corey et al., 1997a). By using an internal RNA standard, differing enough from the wild type target sequence to allow specific detection, the variations in reverse transcription can also be controlled (Verhaegen et al., 1998; Ylikoski et al., 1999).

In the second assay for relative quantification of PSA/hK2 mRNAs, 10^4 copies of internal standard was used. The standard curve was determined by using dilutions of PSA and hK2 cDNA together with 10^4 molecules of IS. The assay was linear from 10^2 to 10^6 molecules. The intra-assay coefficient of variation (CV%) was 9-33% for PSA and 15-28% for hK2 in the concentration range 10^2 to 10^6 mRNA molecules. In this study the same primers were used for PSA, the internal standard

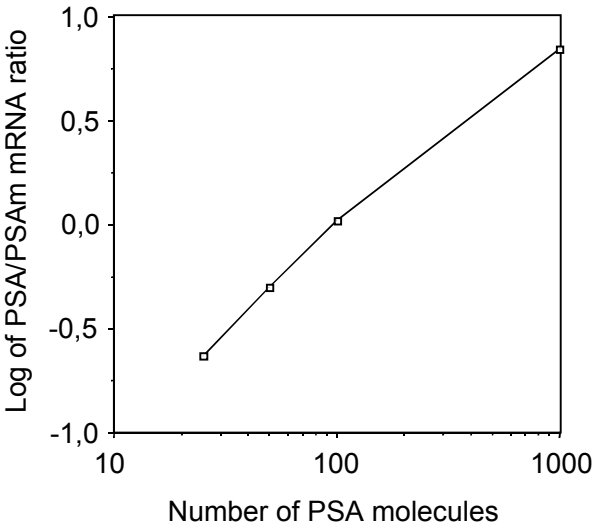

Figure 4. Standard curve of the quantitative reverse-transcription-chain reaction (RT-PCR) assay for prostate specific antigen (PSA). PSAm = mutated PSA (IS).

and hK2, whereas different primer sequences have been used for the target genes in other studies (Ylikoski et al., 2001b; Ylikoski et al., 2002). The mRNAs of PSA and hK2 are similar enough to permit simultaneous amplification with identical PCR primers while still enabling their separate detection. With this type of experimental setup the relative amounts of the two amplification products remain unchanged throughout amplification and reflect the proportions of mRNA transcripts originally present in the sample (Stenman et al., 1999).

The primers used in the second assay amplified wild type NM_001648 (Lundwall and Lilja, 1987) and 6 out of the 13 known splicing variants of PSA mRNA; M21896 (Riegman et al., 1988), U17040 (Monne et al., 1994), AJ459783 (Heuze-Vourc'h et al., 2003), PSA-RP1 (Heuze et al., 1999), AJ310938 (Heuze-Vourc'h et al., 2001) and alternative spliced PSA by Tanaka et al. (Tanaka et al., 2000) giving 315 bp, 458 bp or 757 bp pcr products. Primers amplify also wild type hK2 mRNA BC005196 (Schedlich et al., 1987) and nine out of the ten splicing variants of hK2 mRNA, which are NM_005551.3,

NM-001002231 and NM_001002232 (Olsson et al., 2004), S39329 (Riegman et al., 1991), AF118745 and AF118746, AF118747 (Liu et al., 1999), AY429509 (Michael et al., 2003) and AY429510 (Michael et al., 2003) giving 315 bp, 339 bp, 352 bp or 428 bp pcr products. One splicing variant of hK2, that is AF336106 (David et al., 2002) is not amplified with these primers. Only transcripts of 315 bp from PSA and hK2 were detected by agarose gel electrophoresis.

4.2. CLINICAL SAMPLES

4.2.1 PSA and PSM mRNAs in circulation (I, II, III)

To study if the expression of PSA and PSM mRNAs in blood could be used as a marker for circulating malignant cells, blood samples from PCa patients were analyzed for PSA and PSM mRNA expression (I, II). PSA mRNA was detected in 50% (9/18) of PCa patients with bone metastases, while 7 patients with organ confined disease and 9 healthy controls were negative (Table 8). In other studies 27%-83% of blood samples from PCa patients with metastases and 2%-80% of blood samples of patients with local PCa have been founded to be positive for PSA mRNA. However, in these studies 1%-11% of the control samples were also positive (Moreno et al., 1992; Katz et al., 1994; Seiden et al., 1994; Katz et al., 1995; Cama et al., 1995; Ghossein et al., 1995; Henke et al., 1997; Melchior et al., 1997; Zhang et al., 1997b; Hara et al., 2002; Halabi et al., 2003). In three studies the sensitivity was increased by using nested

Table 8. Clinical characteristics, PSA mRNA, PSM RNA, serum PSA concentrations and treatment of prostate cancer patients (studies I and III).

Age	PSA mRNA	PSM mRNA	Stage	Grade	serum PSA (μg/L)	Bone Scan	Treatment
Metastatic disease							
78	+	nd	T2	G3	0.5	+	estrogen
80	+	+	T3	G2	42.0	+	orc.
66	+	+	T3	G3	85.0	+	RP, orc.,estrogen
69	+	+	T4	G3	99.0	+	TURP, Zoladex
73	+	+	T4	G2	143.0	+	orc., TURP
78	+	+	T3	G2	317.0	+	RP, orc., estrogen
81	+	+	T3	G2	846.0	+	orc.
62	+	+	T2	G3	289.0	+	RP, orc.
64	+	+	nd	G3	1403.0	+	estrogen
57	-	+	nd	G3	0.5	+	orc.
85	-	+	T3	G2	495.0	+	TURP
62	-	+	nd	G3	0.5	+	orc.
71	-	+	nd	G2	74.0	+	orc.
75	-	+	nd	G3	2.7	+	estrogen
65	-	+	nd	nd	nd	+	Flutamide
62	-	-	T3	G3	1180.0	+	orc.
69	-	-	nd	nd	1.0	+	orc., estrogen
67	-	-	nd	G3	36.0	+	Zoladex
Organ confined disease							
81	-	+	T1b	G3	3.3	-	TURP, orc.
79	-	+	T4	G1	47.0	-	orc.
57	-	+	nd	G4	17.0	-	estrogen, Zoladex
73	-	+	T1b	G1	0.5	-	TURP
81	-	-	T3	G1	0.5	-	orc., TURP
66	-	-	T3	G1	nd	-	RP
74	-	-	nd	G3	0.5	-	Zoladex

RP= radical prostatectomy, orc.=orchiectomy, TURP=transurethral resection of the prostate, nd= not determined

PCR with 40+40 cycles, but this decreased specificity dramatically, giving positive results in 40%-100% of the control samples (Smith et al., 1995; Henke et al., 1997; McIntyre et al., 2000).

In the present study negative results in patients with metastatic disease were associated with successful endocrine therapy and low levels of serum PSA, and the correlation between serum concentrations of PSA and the presence of PSA mRNA in peripheral blood was statistically significant (I). PSA RT-PCR positivity has also been found to correlate with capsular penetration, stage and grade as well as to the presence of skeletal metastases (Katz et al., 1994; Cama et al., 1995; Kurek et al., 2004; Ignatoff et al., 1997). A higher risk for recurrence and decreased survival of patients with androgen independent PCa has also been shown to correlate with positive RT-PCR for PSA mRNA (Katz et al., 1995; Ghossein et al., 1997; Hara et al., 2002; Halabi et al., 2003).

PSM mRNA was expressed in 14/17 (82%) PCa patients with bone metastases and in 4/7 (57%) patients with organ confined disease (Table 8). However, 5 out of 6 female controls and all 7 male controls (92% of all controls) were also positive. When the source of the false positive results was studied it was founded to be associated with isolated leukocytes, whereas erythrocytes and platelets as well as four different hematopoietic cell lines (K-562, U-937, HL-60, Jurkat) were negative for PSM mRNA RT-PCR. This demonstrates true expression of PSM mRNA in normal leukocytes, because all control samples without reverse transcriptase as well as RT-PCR reactions without template were negative. In other studies, 48%-95% and 16%-72% of samples from patients with local and metastatic disease were founded to be positive, respectively (Israeli et al., 1994a; Loric et al., 1995; Zhang et al., 1997b; Grasso et al., 1998; Okegawa et al., 1999; Adsan et al., 2002; Varkarakis et al., 2003). In other studies 3%-96% of the control samples have also been found to be positive (Israeli et al., 1994a; Gala et al., 1998; Millon et al., 1999; Llanes et al., 2002; Hara et al., 2002; Schmidt et al., 2003; Chu et al., 2004). In most of these studies both PSM and PSM' were detected as well as PSML in some cases. In one study 6% of the control samples were found to express PSM, while 29 % expressed PSM' (Hisatomi et al., 2002). It was concluded that PSM' may induce false positive results when PSM mRNA is used for diagnosis of prostate cancer. Because PSM is found to be expressed more strongly in cancer than in benign prostatic tissue (Sweat et al., 1998) and the ratio of PSM to PSM' has been found to be increased in cancer (Su et al., 1995; Schmittgen et al., 2003), it may be speculated that primers excluding PSM' might be more specific for studying circulating cancer cells by RT-PCR. In study II the primers of Israeli et al. (1994) were used, and they detected all PSM splicing variants as well as PSML.

The results obtained by PSA RT-PCR confirmed that many patients with prostate cancer have prostatic cells in peripheral blood. However, in half of the patients with advanced cancer treated by orchiectomy or with estrogens, PSA mRNA could not be detected, but in the same samples PSM mRNA was expressed. It is known that hormonal treatment of prostate cancer will decrease the expression of PSA at the protein and mRNA level in prostatic tissue, but the expression of PSM has been shown to be upregulated after androgen-deprivation therapy (Wright et al., 1996), which, together with the fact that we detected PSM expression also in normal blood leukocytes may explain our results.

The results for PSM mRNA in white blood cells and the finding that low levels of PSA mRNA detectable by nested primer RT-PCR is present in blood cells of non-prostatic origin (Smith et al., 1995; Henke et al., 1997; McIntyre et al., 2000) show that the background expression of genes

thought to be tissue-specific is a problem when sensitive nested PCR methods are used to detect cancer cells in circulation.

To control for the background expression, a quantitative RT-PCR method measuring the absolute concentrations of PSA mRNA molecules in patient and control samples was developed. The sensitivity of our assay was high enough to detect PSA mRNA in nearly half of the control samples. It has been estimated that there is one transcribed PSA or PSM mRNA molecule per 100 000 white blood cells and thus there would be 250 transcribed PSA or PSM mRNA molecules in a 5 mL blood sample (McIntyre et al., 2000). This means that the sensitivity of a qualitative assay should be lower in order not to detect these molecules and the detection limit in quantitative assays should be at most 50 copies/mL blood. In study III, all 88 control samples contained less than 26 PSA mRNA copies/mL of blood, and therefore this was used as the cutoff level. In another study using 100 copies/mL as a cutoff level 6% (2/34) of the control samples were positive (Ylikoski et al., 1999), while 5% (1/19) or 23% (3/13) blood samples from BPH patients were were positive (Ylikoski et al., 2002; Emmanouilidou et al., 2003). All the positive control BPH patients had serum PSA levels between 4-10 μg/L while BPH patients with negative PSA mRNA had serum PSA levels below 4 μg/L (Emmanouilidou et al., 2003).

Prostatic surgery or endocrine treatment induced clear changes in the PSA mRNA level in blood. Immediately after RP or TURP, PSA mRNA levels increased in 27% and 29% of the samples, respectively. Orchiectomy and biopsy also caused a moderate increase in 25% and 13% of the samples, respectively. Radical prostatectomy caused a doubling in the number of positive samples in our study, which is similar to results observed in other studies (Oefelein et al., 1999; Ogawa et al., 1999). TURP and biopsy also increased the concentration of PSA mRNA in circulation to the same extent as in earlier studies (Moreno et al., 1997; Heung et al., 2000; Hara et al., 2001; Price et al., 1998). In most cases the PSA mRNA levels dropped below the detection limit within 1-3 days (Figure 5). This shows that prostatic surgery causes a temporary dissemination of prostatic cells into circulation (Figure 6) as has been also shown in other studies (Price et al., 1998; Goldman et al., 1998; Oefelein et al., 1996b). In one case treated by TURP the level remained moderately elevated for 3 months declining thereafter. In one study, post-operative positivity 6-8 weeks after RP has been shown to predict disease progression (Shariat et al., 2003b). Previously Moreno et al. (2001) showed by flow cytometry, that chemotherapy reduced the number of circulating cancer cells in PCa patients (Moreno et al., 2001). Our study is the first to show that the changes in concentrations of cancer cells in circulation can be followed by quantitative RT-PCR.

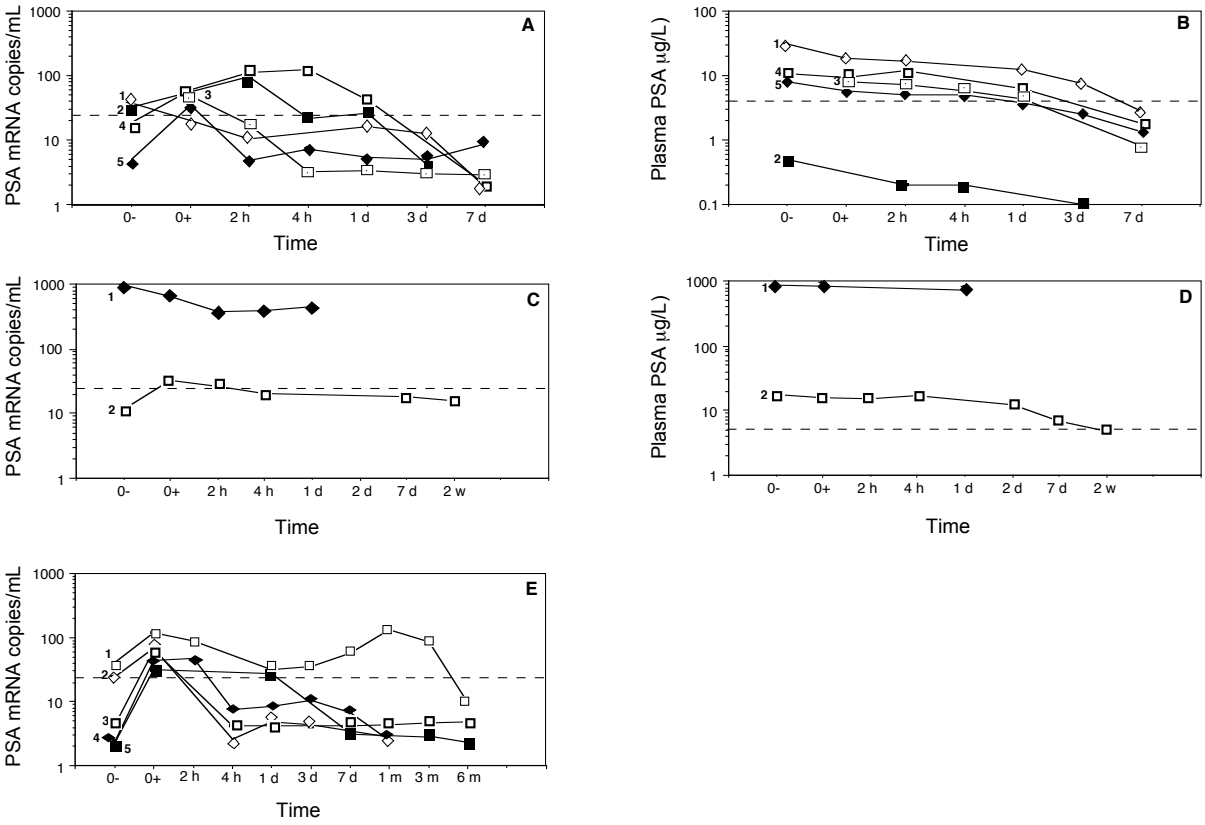

Figure 5. Panel A: Number of prostate specific antigen (PSA) mRNA molecules/mL blood in five postoperatively positive radical prostatectomy patients during 7 days of follow-up after operation. Panel B: Plasma PSA concentrations of patients shown in panel A. Panel C shows the content of PSA mRNA in blood during 2 weeks' followup in two patients subjected to orchiectomy. Panel D: Plasma PSA concentrations of orchiectomy patients shown in panel C. Panel E shows PSA mRNA during 6 months' follow-up in the five patients undergoing transurethral resection of the prostate (TURP). Levels exceeding 25 molecules of PSA mRNA/mL blood were considered positive shown as dash line. Notice that the time-scales are non-linear and vary between the panels. The numbers on the graphs relate to cases mentioned in the text of study III. 0- = preoperative sample, 0+ = postoperative sample.

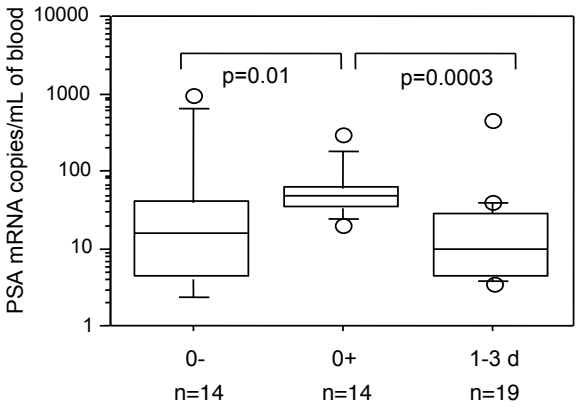

Figure 6. The amount of PSA molecules/mL in PSA mRNA positive samples taken pre- (0-) and postoperatively up to one hour (0+) and 1-3 days after operation (RP, TURP, orchiectomy, total androgen blockade or biopsy).

4.2.2. The ratio of hK2/PSA mRNA in prostatic tissue (IV)

Studies on protein expression by immunohistochemistry have shown that the PSA protein levels are lower in prostate cancer tissue than in benign prostatic tissue. In contrast, the levels of hK2 protein have been found to increase in cancer tissue or to decrease less than those of PSA (Darson et al., 1999; Darson et al., 1997; Magklara et al., 2000). On the RNA level, the expression of hK2 has been found to be increased in prostate cancer tissue, while PSA is decreased or increased

less than hK2 (Qiu et al., 1990; Herrala et al., 2001; Fuessel et al., 2003; Gelmini et al., 2003). In most of these studies the ratio of hK2/PSA appears to increase in prostate cancer compared to benign tissue. We utilized a novel quantitative RT-PCR method to measure the ratio of hK2 and PSA mRNAs in benign and malignant prostatic tissue. This method is designed to accurately quantify the relative expression levels of two homologous gene products in the same reaction tube with the same RT-PCR primers.

PSA and hK2 mRNAs were detected in the normal prostate, in all 13 prostate cancer samples and in 13 out of 14 BPH samples. The range of the hK2/PSA ratios was narrow, i.e., 0.38 – 0.50 in normal prostatic tissue, 0.34 - 1.20 in BPH tissue and 0.46 – 0.91 in cancerous tissue. In LNCaP cells the hK2/PSA mRNA ratio was 0.32. No expression of PSA or hK2 was detected the cell lines DU-145 and PC-3. The median ratio of hK2/PSA mRNA in normal and BPH tissue was 0.43 and 0.44, respectively, while that in WHO grade 2 and 3 prostate cancer tissue was 0.63 and 0.73, respectively. The difference between cancerous and benign tissues was statistically significant, p=0.032 and p=0.035 between G2 tumors and normal or hyperplastic prostatic tissue, respectively, and p=0.006 for G3 tumors compared to normal or hyperplastic prostatic tissue (Figure 7).

Although adjacent pieces of prostatic tissues were subjected to histopathological examination, a limitation of our method is the lack of microscopic control of the specimens extracted for RT-PCR analyses. The samples were obtained during palliative TURP and thus they may contain both cancerous and benign epithelial prostatic cells in various proportions. Thus the true differences in hK2/PSA ratios between cancerous and benign tissue are probably larger than those measured. It would be possible to study the ratio of hK2/PSA expression more accurately by using microdissection of prostatic tissue

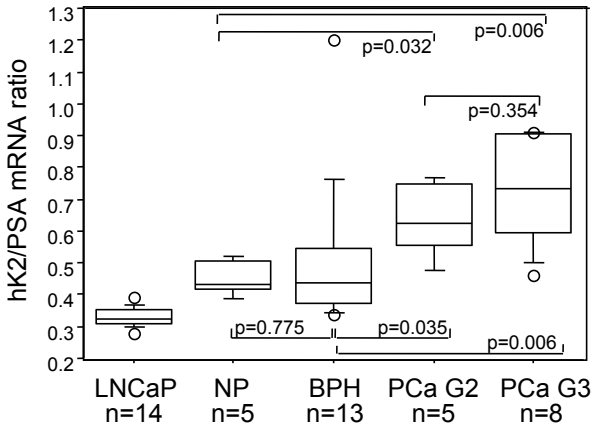

Figure 7. hK2/PSA mRNA ratios in normal (NP) and benign prostate hyperplasia (BPH) tissue and WHO grade 2 (PCa G2) and 3 (PCa G3) prostatic tumors as well as in LNCaP cell line. The box denotes the 25th, 50th and 75th percentiles and the whiskers represent the 10th and 90th percentiles. Circles mark values outside these limits.

under microscopic control. The present study already shows that the method used is suitable for accurate measurement of the relative expression of these gene products and the sensitivity is sufficient for measurement of mRNA in minute samples.

As compared to real-time PCR, the present method has the advantage of much lower variation in the ratio between the gene products measured, for example, 3-fold rather than more than 1000-fold in the ratio of PSA or hK2 to GADPH (Fuessel et al., 2003). Part of the variation in real-time PCR methods is probably caused by variable content of epithelial and stromal tissue, but differences in the amplification of the target genes probably contributes to the variation. The expression levels of GAPDH and other housekeeping genes have shown to be very variable, more than 30-fold in human blood mononuclear cell culture and whole blood (Dheda et al., 2004).

The mechanism causing differences in expression of hK2 and PSA are not known but various theories have been presented.

Studies on the copy number of the PSA and hK2 genes in tumor tissue have suggested amplification of hK2 as a possible cause (Herrala et al., 2001), but because the genes are so closely located (Diamandis and Yousef, 2002), it is unlikely that amplification of only one of these is a common phenomenon. No mutations in the PSA gene have been found in malignant prostate tissue (Baffa et al., 1996). The expression of hK2 and PSA are thought to be coordinated and regulated by common and gene-specific enhancers (Wei et al., 1998), but this is not supported by experimental evidence (Herrala et al., 2001). Thus the mechanisms causing the change in expression levels of PSA and hK2 remain to be explained.

Changes in expression of PSA and hK2 in cancer may have implications for the behavior of the tumor. In recent studies PSA has been shown to cause degradation of gelatin and type IV collagen in Matrigel and activation of progelatinase A (MMP-2) in a cell-free system (Pezzato et al., 2004). It has also been shown that Zn(2+) in the human prostate may suppress the invasion and metastasis of prostate cancer cells through inhibition of the proteolytic activity of PSA (Ishii et al., 2004). In prostate cancer the Zn(2+) levels are markedly decreased as compared to normal surrounding tissues (Zaichick et al., 1997), suggesting that high concentrations of zinc in prostatic tumors might even inhibit tumor growth by inhibiting PSA activity. On the other hand, PSA has been shown to exert antiangionenic activity (Heidtmann et al., 1999; Fortier et al., 2003). Thus a reduction in PSA expression would be compatible with increased aggressiveness. HK2 is a highly active trypsin-like proteinase (Bourgeois et al., 1997; Lovgren et al., 1999), which has the potential of participating in proteolytic cascades contributing to invasive tumor growth, (Frenette et al., 1997; Mullins and Rohrlich, 1983). These findings suggest that increase in the ratio of hK2/PSA expression may favor aggressive tumor growth, but direct evidence for this is lacking. Our results showing an increased hK2/PSA mRNA ratio in prostate cancer compared to benign prostatic tissue support this hypothesis.

5. CONCLUSIONS

We have shown, that PSA mRNA can be found in circulation of patients with metastatic prostate cancer and that negative results in patients with metastatic disease are associated with successful endocrine therapy and low levels of serum PSA. No expression was found in negative control samples, but when the sensitivity of the method was increased by increasing the number of PCR cycles, almost half of the control samples became positive. We therefore developed a quantitative RT-PCR method and were able to set a cutoff limit at which all control samples were negative. With this method we found that the level of PSA mRNA molecules in peripheral blood increased after prostatic surgery, suggesting a temporary dissemination of prostatic cells. After the initial increase the PSA mRNA levels decreased. However, the preoperative levels only occasionally correlated with other prognostic indicators such as serum PSA, TNM classification, grade and Gleason score. Thus our results add further support to those of many other studies showing that quantification of PSA mRNA expressing cells in circulation of cancer patients does not provide reliable prognostic information in prostate cancer patients.

We were able to show that PSM mRNA is found in circulation of patients with metastatic PCa, who had negative result for PSA mRNA. Androgen ablation is known to decrease the expression of PSA in prostatic tissue while the expression of PSM is upregulated and this might explain these results. However, we detected PSM expression also in normal blood leukocytes. Additionally, the primers used will amplify both PSM and its splicing variant PSM', which has been shown to increase the frequency of false positive results in control subjects. The use of primers specific to PSM' might have reduced this problem. Another method, which might reduce the false positive results is a quantitative RT-PCR measuring the ratio of PSM/PSM' mRNAs. This ratio has been shown to be increased in cancer (Su et al., 1995).

Using an accurate quantitative RT-PCR for measurement of the relative levels of hK2 and PSA we showed that the ratio of hK2/PSA is increased in prostate cancer when compared to benign prostatic tissue. Earlier studies have shown this phenomenon also on the protein level. The mechanism causing the change in expression is not known. However, PSA has been shown to exert antiangionenic activity, and thus a reduction in PSA expression would be compatible with increased aggressiveness. hK2 is on the other hand a highly active trypsin-like proteinase with the potential of participating in proteolytic cascades contributing to invasive tumor growth. These findings suggest that a change in the ratio to hK2/PSA expression may favor aggressive tumor growth. Thus our method is of potential utility for evaluation of the aggressiveness of prostatic tumors.

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